Reproductive biology, ultrastructure and molecular characterisation of Liposcelis bostrychophila (Badonnel) (Liposcelididae: Psocoptera) and its intracellular rickettsial endosymbionts.

Yusuf, Mohammed Abdi Sheikh

King's College London

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Mohammed Abdi Sheikh Yusuf

University of London
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DEDICATED TO MY DEAR MOTHER AND GRANDMOTHER

ABSTRACT

The psocid *Liposcelis bostrychophila* is a stored product pest and has an interesting and complex reproductive biology. However information available on this insect is rather limited. This thesis describes the gross morphology, anatomy of the reproductive organs of *L. bostrychophila* and to a lesser extent the digestive system, using serially sectioned materials for light microscopy.

This study also involves an investigation of the fine ultrastructural details of the reproductive organs of adults and their developing juveniles.

~~*bostrychophila*'s~~ oocyte and ~~oogenesis~~ were also studied. The electron microscope (EM) pictures of the ultrastructure of psocid gonads revealed the presence of endosymbiotic rickettsiae belonging to the α-proteobacteria. The implications of the presence/absence, location, mode of transmission and the ultrastructure of the *Rickettsia*-like organisms (RLO) found to inhabit in the ovaries and other specialised sub-cutaneous structures in this psocid are explored.

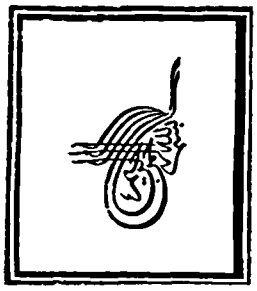
Transovarial transmission of these bacteria has been also established, by conducting egg-embryo, juvenile and adult ultrastructural evidence using EM.

The standard technique of demonstrating the significance and the possible role of these endosymbionts' relationship with the host consists of eliminating the microrganisms, resulting bacteria-free individuals termed aposymbiotic. To achieve this a range of antibiotic and heat treatments were employed. One antibiotic (rifampicin) produced aposymbiotic individuals, whilst the prolonged and repeated exposure of high temperature and tetracycline achieved a gradual decontamination of the psocid infection as validated by EM micrographs.

The nature of the *L. bostrychophila* rickettsial endosymbionts were further characterised using PCR to amplify the 16S r-RNA, a widely recognised and conserved bacterial gene. The PCR product was characterised using restriction and sequence analysis. This provided the genetic characterisation, phylogenetic and nucleotide variation analysis of bacterial endosymbionts, without the necessity of culturing the bacteria independently of the host. The sample sequence when compared With the bacterial genome database, the sample sequence was seen to be most similar to those from the rickettsias and their relatives. There was 97.6% similarity in the nucleotide sequence from the liposcelid isolates and intracellular parasitic bacteria found in many other insects.

And finally the variability in egg production of *L. bostrychophila* populations from different locations in the UK was quantified. These populations represented a sample of the variability that allows this insect to become established and exploit a wide range of habitats. The relationship between the variation in egg production of these populations (30) and bacterial load were explored. Different population of *L. bostrychophila* were found to harbour different bacterial loads. This two factors were found to negatively correlate. The interaction and influence of bacterial density on the overall fitness and survivorship of different populations of *L. bostrychophila* is studied.

ACKNOWLEGEMENTS



In the Name of Allah, Most Gracious, Most Merciful

Verily, all things have we created in proportion and measure.
Qur'an: Sura *Qamar* (LIV), Verse 49.

For we do determine; for we are the best to determine (things).
Qur'an: Sura *mursalat* (LXXVII), Verse 23.

Thanks to Almighty Allah in allowing me successfully complete this work.

****

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

APPENDIX CONTENT

CHAPTER ONE
GENERAL INTRODUCTION

CHAPTER ONE

1.0. GENERAL INTRODUCTION

The Psocids belong to the insect order Psocoptera, a worldwide order of common cryptic small to minute insects, currently with over 4000 species in 35 families (Smithers and Lienhard, 1992; Lienhard 1996 *pers. com.*). Psocids are soft-bodied insects, usually less than 6mm in length, which live in variety of terrestrial habitats. They are geographically widespread and able to exploit a range of food materials from microepiphytes to human stored food. Most psocids are bark, foliage or tree-dwellers but rarely injurious to the plants. Psocids, one of the minor orders, are relatively readily dispersed, and found throughout the world (New, 1987). Both winged and wingless psocids can be carried by the wind (Freeman, 1945). Human involvement in transportation and dissemination of mainly wingless ones also contributed the expansion of psocid range.

Psocids are hemimetabolous, pterygote insects which normally pass through up to six nymphal instars. In some psocid species, including the one on which this study is based, the number of instars is reduced to four. This has been alternatively interpreted as being either an advanced character Smithers (1972), or a primitive one New (1987). It is generally believed that the Psocoptera diverged from some basal hemipteroid stock (New 1974) in the beginning of the Permian period (270 million years ago). Psocopterans are considered to be somewhat intermediate between the Zoraptera (Orthopteroid) and the remaining hemipteroid orders. The closest relatives to Psocoptera are thought to be the chewing lice (Mallophaga) and the sucking lice (Anoplura) (Gullan & Cranston 1994). They share certain morphological features with Mallophaga (e.g. mouthparts). The Mallophaga are believed to have evolved directly from Psocoptera. It would appear to represent a relatively small evolutionary step for Psocoptera inhabiting bird nests to transfer onto birds, and from that stage, to evolve additional traits characteristic of ectoparasites (Kim and Ludwig, 1982). Sharing such specialised organs as the sitophore sclerite and sclerotised filament in the hypopharynx, pick-like lacinia and the polytrophic ovarioles (Richards and Davies, 1977), all indicate their common psocopteran-like stock.

These three taxa (orders Psocoptera, Anoplura, and Mallophaga) are grouped together in the superorder Psocodea by some authors, with the Thysanoptera and Hemiptera forming sister groups. Morphological accounts of Psocoptera were given by Badonnel, 1948, 1951; Smithers, 1972a; Gunther, 1974; New, 1974; Lienhard, 1999. Seeger (1979) considered psocids a monophyletic group in his study on eggs and hatching mechanisms.

1.1. Psocid classification

The order Psocoptera is divided in three sub-orders: Trogiomorpha, Troctomorpha and Psocomorpha, with general acceptance that the sequence represents evolutionary advance. The sub-order Psocomorpha is the largest suborder consisting of more than 22 families, the largest of which is the Psocidae with many bark-inhabiting species throughout the world. Four families are included in each of the other two suborders; Troctomorpha- Amphientomidae, Liposcelididae, Pachytroctidae, and Sphaeropsocidae; Trogiomorpha - Lepidopsocidae, Psoquillidae, Psyllipsocidae, and Trogiidae.

The classification of the order is comprehensively summarised by Smithers and Lienhard (1992) and Smithers has also produced a bibliography of the Psocoptera (Smithers, 1965a) and a catalogue of world species (Smithers, 1967).

The suborder Permapsocida was also suggested for fossil psocids from Permian period (Tillyard, 1926, 1935). The Oligocene material from amber (Enderlein, 1911; Mockford, 1969; Vishniakova, 1975) however is similar to present-day psocid families. Most of the present-day psocids have little impact on humans (Turner and Ali, 1996). They are predominantly outdoor species with well-developed wings, frequently occurring on the bark or foliage or trees and shrubs, under bark or stones or in dead leaves, hence the common name 'barklice'.

The outdoor species feed on organic matter, including fungi, algae, lichens, pollen, and fragments of decaying organic material (Borror *et.al.*, 1989; Romoser and Stoffolano, 1994).

Only a few psocid species are found indoors and these are mainly wingless. The wingless psocids may reach large enough populations among books, papers, stored cereal grains,

insect collections, and other materials to constitute a pest situation. Wingless psocids belong to one of three families, namely the Trogiidae, the Psyllipsocidae and the Liposcelididae. The family Trogiidae includes the genera *Lepinotus* and *Trogium*, both common domestic psocids of temperate areas. They are also found in houses, barns and granaries, primarily of importance as contaminants of cereals and packaging materials (Finlayson, 1949; Gurney, 1950; Fahy, . 1971).

The genus *Psyllipsocus* (Selys-Longchamps), in the Psyllipsocidae is also associated with domestic situations and is cosmopolitan (New, 1974), although relatively uncommon.

Some other winged genera are often attracted indoors by light. These include the genera *Lachesilla* (Westwood) [Lachesillidae], *Ectopsocus* (McLachlan) [Ectopsocidae] and *Peripsocus* (Hagen) [Peripsocidae]. Their ability to fly enables them to occur in a wider variety of destinations than do flightless psocids. Though mainly a nuisance, some species like *Ectopsocus pumilis* are capable of injuring grain in storage and may feed on other starch materials (Gurney, 1950).

The genus *Liposcelis* (Motschulsky), in the family Liposcelididae, (commonly called booklice) is probably the single most economically important in the Psocoptera (Mockford, 1996 *pers. com.*).
The vast majority of psocid related pest problems in the UK and elsewhere are due to members of this genus (Turner, 1987, 1989, 1994, 1996, 1998; Turner and Ali, 1996; Pike, 1994; Rees, 1994b; Leong and Ho, 1995).

1.2. Geographical Distribution of the Psocoptera

Knowledge of the geographical distribution of the Psocoptera is limited by the locations where specialist collections have been made and analysed (For example, Broadhead, 1950; Badonnel, 1951; Turner, 1987, Lienhard, 1990; Baz, 1988 , 1989 for Europe and Mediterranean: Smithers, 1965; Thornton, 1965; New, 1971, for Australasia (Oceania): Badonnel, 1931, 1935, 1936 , 1943, 1975 ; Broadhead and Richards, 1982 for African psocoptera: Badonnel, 1977 ; Turner, 1975 , 1976 for Caribbean psocids: Mockford, 1963, 1979, 1987b; Garcia-Ald rete, 1974 , 1983, 1988 for north and south America respectively).

The geographical patterns of the Psocoptera range from species which are cosmopolitan (*Liposcelis bostrychophila* (Badonnel)) to groups which have limited or highly disjunct (dispersed) distributions. Some closely related psocids apparently have separate centres of radiation (New, 1975). The most widely distributed psocids are predominantly the wingless ones such as species of *Liposcelis*, *Lepinotus*, and *Trogium*. Most of these psocids are to some extent associated with domestic situations and like many other storage pests are being carried by man mainly through transport of foodstuffs and plant materials.

This work is concerned with *Liposcelis bostrychophila*, probably the most cosmopolitan of psocid species.

1.3. The genus *Liposcelis*

All liposcelids are wingless, dorsoventrally flattened insects measuring between 0.7 and 1.5 mm. They are characterised by a broad and flattened hind femur. The genus *Liposcelis* contains about 100 species found in various habitats ranging from birds nests to soil and stored products of vegetable origin (Lienhard, 1990). At least 10 species of this genus are found as pests in granaries and household situations (Mockford, 1979). In the UK there is a total of 14 *Liposcelis* species of which 8 are of some economic importance (Table 1.1).

There has been a long history of liposcelids recorded as pests of households (Howard & Marlett, 1902; Harper-Grey, 1917; Sikora, 1918; Back, 1920), although the identity of these species described in these early papers is often uncertain. The species of *Liposcelis* are difficult to separate taxonomically and in the past there was a tendency to call all domestic liposcelids *L. divinatoria(um)*, the common booklouse. Thus leading to a considerable uncertainty as to which species is being studied (Turner, 1994). *L. bostrychophila (bostrychophilus)* was referred in the literature as *L. divinatoria(um)* (Ghani and Sweetman, 1951), *Troctes divinatoria*, *L. granicola* (by Broadhead and Hobby, 1944a, a synonym of *L. divergens* Badonnel, described in 1943 in *Faune de France*- this work reached England two years later). The situation for the palaearctic liposcelids has been clarified by Lienhard (1990), who gives a detailed taxonomic

revision of the 34 species found in the region. Although Lienhard's recent revision has immeasurable value it is by no means complete.

Broadhead (1950) and Gunther (1974) in earlier revisions of genus *Liposcelis* tended to create 'super species' consisting of complex assemblages of morphologically similar liposcelids. Species within these assemblages are often difficult to distinguish. The unequivocal identification of single individuals is sometimes impossible, constituting the liposcelid species complex (e.g. *bostrychophila* species complex, *silvarum* species complex (Lienhard, 1990).

Lienhard (1990) divided the Palaearctic members of the genus *Liposcelis* into four distinct sub-groups as identified by Broadhead (1950), using a range of morphological characteristics including Chaetotaxy of the prothoracic lobes, pro- and meso-sternum, abdominal terga 8-10 and the shape of the gonapophyses. These sub-groups are:

Group IA (*entomophila* species complex): i.e. *L. brunea, platina, albothoracica and entomophila.*

Group IB: (*silvarum* species complex): i.e. *Liposcelis myrmecophila, meridionalis, priesneri, bicolor, picta, pulchra, uxoris, keleri, silvarum, kyrosensis, orghidani, rufa, pearmani,* and *decolor.*

Group IIC: (*mendax* species complex): i.e. *L. formicaria, pubescens, paetula, mendax,* and *obscura.*

Group IID (*L. bostrychophila* species complex): i.e. *paeta, corrodens, arenicola, rugosa, tricolor, semicaeca, compacta, minuta, edaphica, bostrychophila* and *aconae.*

Very little morphological difference exists within some of the species complex. Work is now underway to investigate the relationship between these morphological characters and the insect phenotype, using enzyme polymorphism of the genus *Liposcelis.*
Turner and De Villiers (unpublished).

Liposcelis species	Mode of Reproduction	Pest situation in the UK	Europe	Tropics
*L. brunnea**	Bisexual	FS	FS	HH/FS
L. entomophila#	„	FS	FS	FS/HH
*L. pearmani**	„	FS	FS	HH
L. decolor#	„	FS	FS	FS
*L. pubescens**	„	FS	FS	HH
L. mendax	„	FS	FS/HH	HH/FS
L. corrodens#*	„	FS/HH	FS	HH/FS
L. bostrychophila#*	Parthenogen	HH	HH	FS
L. obscura	„	FS	FS	FS
L. albothoracica	Bisexual		FS	HH/FS
L. bicolor	„			HH
L. myrmecophila	„			
L. paeta#	„		FS/HH	HH/FS
L. paetula	„			HH

Table 1.1 The list of liposcelid species in the UK together with their pest status. Data from Lienhard (1990) and Turner (1994). FS, food stores; HH, household. * Common UK species # Important tropical species.

1.4. Distribution of Liposcelids

The genus *Liposcelis* is well represented in all zoogeographical areas (Fig. 1.1), although relative to the rest of the world, the Oriental and Australasia representation is reduced (Lienhard, 1990).

The most widely distributed liposcelids are: *L. bostrychophila, corrodens, decolor, entomophila, mendax, obscura, paeta, pearmani, pubescens, rufa, albothoracica, arenicola, bicolor, paetula and tricolor* which are more or less regularly found in domestic situations and food manufacturing/storage facilities. The wide distribution of a relatively small number of psocid species found in either domestic situation or storage facilities is indicative of the role of human involvement in spreading these insects.

1.5. Liposcelids as pests

Liposcelids have been recorded from a wide range of substrates (Turner, 1987) but are primarily a pests of food products, ranging from grain to powdered milk. There is an apparently unique situation in both continental Europe and UK where liposcelids are a particular problem in domestic situations (Fig. 1.1). In the tropics, on the other hand liposcelids mainly affect grain stores.

In the UK there are 51 psocid species of which 36 have been introduced in foodstuffs and other imported materials (Broadhead, 1964). There are 14 liposcelid species in the UK, of which all but one are thought to be introduced (Broadhead *Loc. cit.*), and of these 5 are common in domestic or storage facilities (Turner, 1994). In addition two trogiids, *Trogium pulsatorium* and *Lepinotus patruelis*, are also minor storage pests in the UK.

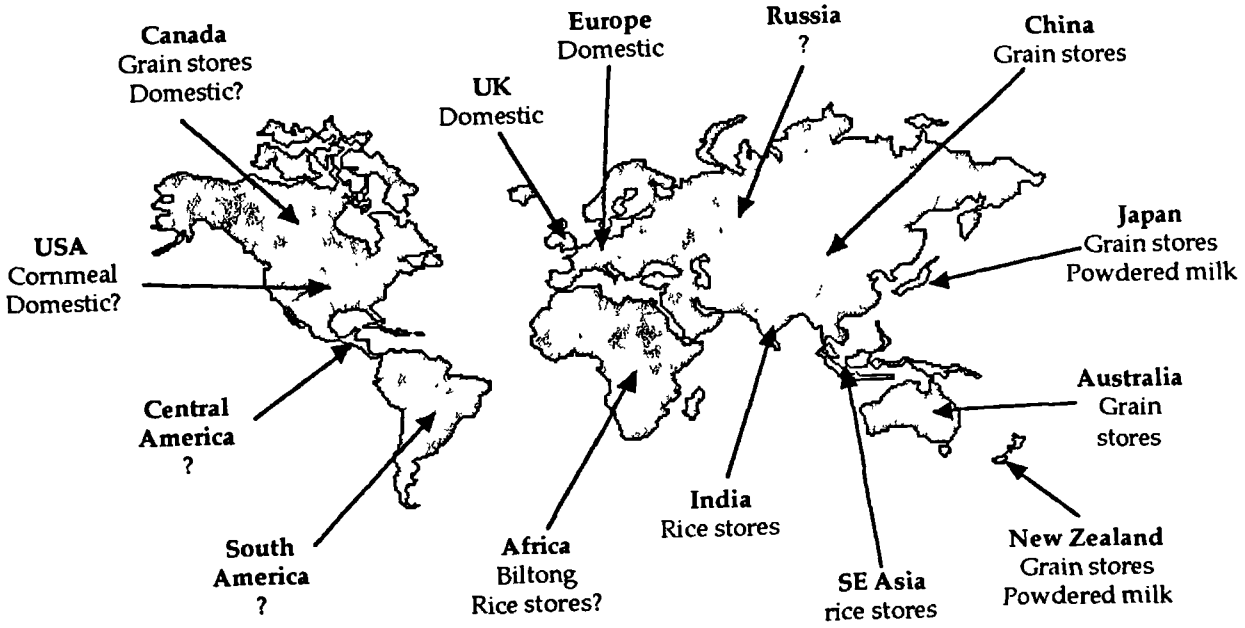


Fig. 1.1. Geographical distribution of recorded psocid related psocid pest problems (Turner 1999).

The economic importance of psocid species worldwide is largely governed by the species involved and locality. For example *L. entomophila* is serious pest of grain storage facilities in the tropics (Rees, 1994; Pike 1994; Leong and Ho, 1995). In the UK however *L. entomophila* is relatively uncommon due to the cooler climate (Turner and Ali, 1996). *L. bostrychophila* on the other hand constitutes more than 90% of all household infestations by psocids in the UK.

Factories and warehouses are colonised by other species (Turner, 1994). The pattern of consumer complaints of infestations by *L. bostrychophila* in the UK is characteristically seasonal (Turner, 1986, 1998; Turner and Maude-Roxby, 1989) peaking in the autumn months. Somewhat similar trends are also observed in psocid related complaints elsewhere in Europe (Turner, 1986, 1987).

L. bostrychophila is classified as being among the most physiologically adaptive pests of stored products. Using the climate plasticity index of Sinha (1991), Turner (1994) calculated a plasticity index value (I_p) of 635 for *L. bostrychophila*, placing it among the most cosmopolitan of storage pests like the flour beetle *Tribolium castaneum* (I_p of 700). The high plasticity of *L. bostrychophila* explains how this insect, though tropical in origin, can also become a serious pest in temperate parts of the world.

The pest status of *Liposcelis* falls into one of four categories.

1. Food Contamination: Apart from grain, where they may constitute a major problem, liposcelids have been found to affect flour, milk and about 50 other foodstuffs (Broadhead and Hobby, 1944a; Lienhard, 1990; Turner, 1987, 1994). Damage may either be due to the direct consumption of the product or more importantly tainting the product with faeces and body parts.

In warehouses, granaries and in ships, they become most conspicuous for here they can breed undisturbed in the warm atmosphere, unhampered by natural enemies and provided with unlimited food supplies in the form of yeast and moulds which grow on the grain if it becomes slightly damp (Broadhead and Hobby, 1945; Haines, 1982; Haines and Pranata, 1982). The amount of foodstuff eaten by the infesting psocids is relatively small, but because of their presence and possible contribution to the gradual heating, they considerably depreciate the market value of these products (Champ and Smithers, 1965).

2. Damage: Liposcelids thrive on 'Stramit'™ a compressed strawboard used for building houses. Occasionally these insects are found in very large numbers (millions) in newly built houses where 'Stramit'™ has been used for partitioning walls (Turner, 1996). This type of infestation is now controlled by impregnating the building material with chemical insecticide.

Liposcelids can also become museum pests as they feed on and destroy dry and preserved insect and herbarium collections.

3. Health Implications: Liposcelids like *L. bostrychophila* are common household pests and have been shown to elicit allergic responses in some people (Spieksma and Smits, 1973; Gutman, 1980; Rijckaert *et. al.*, 1981; Wyniger, 1985; Turner, 1996; Musken *et.al.*, 1998). The significance of booklice as regards to their contribution to the allergen content of house dust has been given elsewhere (Rijckaert *et.al.*, 1981; Musken *et.al.*, 1998).

L. bostrychophila is a potential health risk. It stimulates the production of antibodies in sensitive individuals. A recent trial by Turner *et.al.*, 1996 in the UK on human atopic patients, dermally challenged with psocid antigen, showed that 13 patients out of 250 (5%) had a strong positive reaction to the liposcelid allergen. They concluded that because of the high immune reactivity to psocid antigen, psocids can be considered an important source of allergen. Supporting evidence also exists on the continent (Rijckaert *et.al.*, 1981; Wyniger, 1985; Musken *et.al.*, 1998) showing psocid antibodies in susceptible individuals in Switzerland and Germany. The recent German study by Musken *et.al.* (1998) indicates that as much as 30% of the population carries IgE antibodies for *L. bostrychophila.*

4. Entomophobia: Liposcelids are louse-like in appearance and may cause public panic. The term "Lice" in the name "booklice" or "barklice" is somewhat misleading, for none of these insects are parasitic. The species occurring in buildings rarely cause much damage, but are frequently perceived to be a nuisance.

The earliest scientific record available referred to psocids as "paperlice" (Blankaart 1688, 1690); later the name "dustlouse" was adopted by some authors in Europe. Among the most economically important liposcelids are *Liposcelis corrodens* found throughout Europe, *L. paeta* found in Australia and *L. pearmani(= kideri)* (Broadhead and Hobby, 1944b; Champ and Smithers, 1965; Lienhard, 1990). All are found in various domestic and grain storage facilities (Broadhead and Hobby, 1944a; Broadhead, 1950; Fisher, 1985; Leong and Ho, 1995). *L. entomophila* is of considerable importance in grain

storage facilities in tropical countries (Haines, 1981, 1995; Pike, 1994; Rees, 1994a,b; Leong & Ho, 1995). *L. bostrychophila, L. paeta* and *L. entomophila* are particularly important in the tropics. Although a storage pest in the tropics *L. bostrychophila* is the single most important *Liposcelis* species in Europe and is the major cause of consumer complaints about flour products (Turner, 1987,1994; Turner and Maude-Roxby, 1989). A wide range of mainly farinaceous products may be eaten by this psocid (Turner, 1987; Turner, 1994).

Liposcelis bostrychophila is frequently reported as a household pest in the UK and pests of grain stores elsewhere (Shires, 1982; Turner, 1987; Turner and Maude-Roxby, 1987; Richards, 1991).

1.6. Psocid control

Various approaches have been explored to control psocids (Turner, 1988), of which physical methods were most effective. However these are often not practical in domestic or retail premises (Turner and Maude-Roxby, 1988; Foulk, 1990). Insecticides are the most commonest form of control against psocid infestations but unlike other stored product pests, psocids are not easily killed with insecticide. Pyrethroid based insecticides are not effective against psocids (Pinniger, 1984). Their flattened and small body size enable liposcelids to crawl into narrow crevices which reduces the chances of a psocid coming in contact with enough insecticide dose to kill it. The only paper which comprehensively deals with experimental evaluation of different types of insecticides against *L. bostrychophila* in laboratory conditions is that of Turner *et.al.*, 1991. This study confirmed the earlier observation by Pinniger (1984) and showed that liposcelids are not very susceptible to carbamates and synthetic pyrethroids. This study also showed that the efficiency of pyrethroids can also be enhanced using the synergist- piperonyl butoxide. Very few records of any other forms of control (for example biological control) against liposcelids exist. Attempts to infect *L. bostrychophila* with microbial agent *Bacillus thuringiensis* failed (Turner, 1988). Similar trials made in controlling liposcelids using entomopathogenic fungi have been unsuccessful (Pike, 1987). Buchi (1994) tested two Insect Growth Regulators (IGRs) namely, methoprene and fenoxycarb on *L.*

bostrychophila to disrupt juvenile development and adult fertility. Both IGRs drastically reduced the psocid population growth albeit in higher concentrations that would be used for other storage insect pests.

Predation by *Cheyletus malaccensis* on *L. entomophila* was also observed (Haines, 1995).

1.7. Psocid biology

The number of psocid species is relatively small compared with other major insect orders. Since they have limited impact on humans, they have received limited attention from entomologists. In recent years there has been an increased effort on the taxonomy of the Psocoptera and the numbers of described species has risen dramatically. Nevertheless there are still many more species to be described.

Steyskal (1973) attempted to predict growth in the number of described psocid species and suggested that the number would stabilise by 1974. Lienhard (1999) re-examined the psocid data recently and found that the discovery of new species in both Europe and the rest of the world was still growing in an exponential fashion (Fig 1.2.).

The figure shows the two models, Steyskal (1973) and Lienhard (1999). This increase in the knowledge of the Psocoptera is however almost entirely taxonomic and little information is available on aspects of psocid life since few psocid species have had their biology, ecology or phylogeny studied in detail. Some psocid species, particularly those who feed on dried stored food are ideal experimental animals. Psocids are readily available (a single individual can start an entire colony in the case of the parthenogenetic *L. bostrychophila*), inexpensive to maintain and are have short life cycle.

The limited data available on psocid biology are widely scattered (Smithers, 1967; New, 1987, 1974; Turner, 1994).

The bulk of this information deals with population dynamics i.e. the effect of temperature and humidity on egg production and life histories (Finlayson, 1949; New, 1969 a, b, 1987; Fahy, 1971; Leong and Ho, 1990,1995; Fisher, 1985; Rees and Walker, 1990; Turner and Ali, 1993; Ali, 1994). Very little has been done on their physiology, except water vapour uptake from the atmosphere (Rudolph, 1982). Srivastava and Sinha (1975, 1981 a, b) examined some aspects of the digestive physiology of psocids: specifically the pH and food passage in the gut of the *L. entomophila* as well as its food preference.

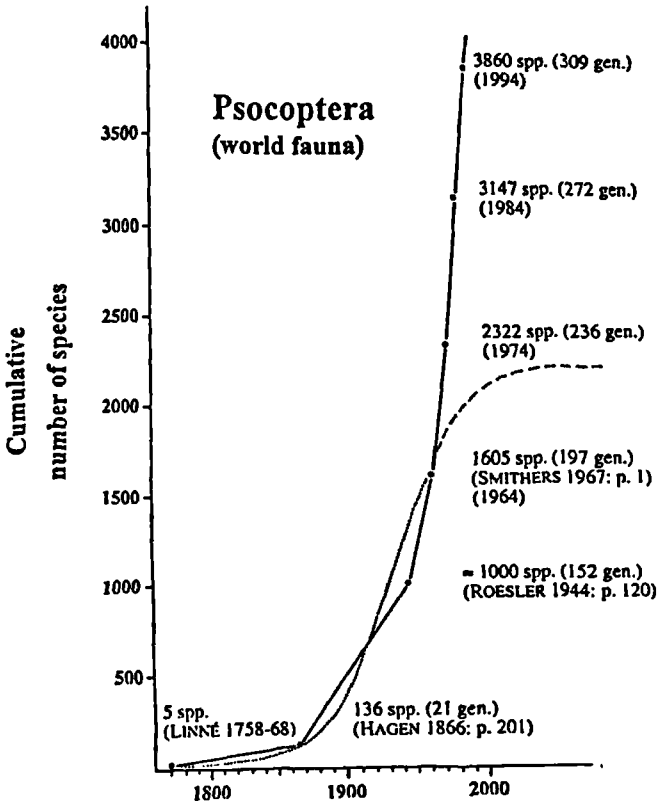


Fig.1.2. The growth of number of described psocid species throughout the world. The dotted line corresponds the prediction of Steyskal (1973). Data from Lienhard (1999).

Psocid morphology is better understood and has been used extensively for taxonomical purposes e.g. Noland, 1924; Badonnel, 1934,1951; Wong, 1970; Wong and Thornton, 1968). Details of internal structures of psocids are less well known.

The most extensive study was by Badonnel (1934) and more recently there have been several papers on specific organ systems e.g. Wong (1970) for internal reproductive organs, Rudolph (1982) on the anatomy of the head and the organs directly involved in active vapour uptake, and Goss's (1954, 1953) anatomy of booklouse reproductive organs and embryology. Little description of other *Liposcelis* species anatomy exists. The scant current knowledge of psocid anatomy is based on descriptions of specific psocid species.

Against this patchy background on liposcelid biology, this thesis aims to focus on some aspects of the reproduction biology of the parthenogenetic liposcelid *L. bostrychophila*, with the data on structure, ovarian development and oogenesis from Goss (1952, 1954), being used as a starting point.

1.8. Sex determination and chromosome numbers in psocids

Sex determination in insects has been extensively studied in many insects and is the subject of many reviews (e.g. White 1954; Goldschmidt, 1955; Doutt, 1959; Smith, 1960; Gowen, 1961; Kerr, 1962; Engelmann, 1979) and more recently by Hunter and Godfray (1995) in wasps, King (1970) in *Drosophila*, Blackman (1980) in aphids, Crowson (1981) in Coleoptera and Lauge (1985), King and Buning (1985) in other insects.

The sex determination in psocids in general is XO. The Kariogamy/types and chromosome behaviour in parthenogenetic psocids remain unexplored. This is partially due to lack of a visible centromere on psocid chromatids (Mockford, *per.com*). The chromosomes of psocids are also short and small, making it impossible to interpret their morphology from standard microscopical preparations (Wong and Thornton, 1966).

The first study on psocid chromosomes was carried out by Boring (1913). He has described in detail the behaviour of the chromosomes of *Cerastipsocus venosus* (Kolbe). The chromosome number of this species in the family Psocidae was established as 2n = 16+1 in the male. The presence of an odd chromosome was subsequently confirmed as being of common feature of all other psocid species investigated thus far (Wong and Thornton, 1966).

Wong and Thornton (1966) examined twenty-one species of psocids representing seventeen genera in twelve families of Psocoptera by squashing testes. They confirmed that the diploid number of chromosome of all psocid species examined was a constant number of 2n=16+1 (19 of 22 species) in all sub-orders. Two species belonging in the sub-order Trogiomorpha, *Psoquilla marginepunctata* (Hagen) with 2n=18+1 and *Psocatropos* sp. Psyllipsocidae) with 2n=28+1 and *Seopsis* sp. (Amphientomidae) in the Troctomorpha with 2n=14+1 are the exceptions.

The chromosome counts of sexual psocids are readily obtained from testis squashes (Boring, 1913; Wong and Thornton, 1966), but cytological investigations of the chromosome bases of parthenogenetic psocids were hampered by lack of suitable method of determining the number of chromosomes in parthenogenetic psocids (Jostes, 1975).

Only two chromosome counts for parthenogenetic psocid species have been reported. The chromosome number of the obligatory parthenogenetic species *Caecilius flavidus* was found to be 2n=18 (Meinander, . 1974). This does deviate from the model

chromosome number for the subgroup Psocomorpha. The other obligate thelytokous species in which the chromosome number has been estimated is *L. bostrychophila* with 2n=18 (Goss, 1954). In a later meticulous study by Jostes (1975) this estimate was revised, giving a somatic chromosome count of 2n=16.

1.9. Biology of *L. bostrychophila* (Badonnel)

Liposcelis bostrychophila has interesting and complex reproduction biology. However information on its biology is limited. This stands in stark contrast to its commonness and importance as a household and stored product pest. What information is available is either widely scattered or confused by the dubious and sometimes erroneous classification and nomenclature, with all species being lumped together as *L. divinatorius. Liposcelis bostrychophila* was first described by Badonnel (1931) from tropical Africa. *Liposcelis divinatorius* was considered as *nomen dubium* by Lienhard (1990) in his revision of genus *Liposcelis* due to its widespread and indiscriminate use.

Goss's paper (1954) on ovarian development and oogenesis in the booklouse was based on a species referred as *L. divergens*. While the information in it remains useful some independent research is justified to determine whether or not the psocid species Goss referred was *L. bostrychophila.* In addition advances in equipment and methodologies in the intervening forty years should allow greater detail and insight into the reproductive process in *L. bostrychophila.* The primary intention of this study is to narrow down some of the apparent gaps in our knowledge by shedding light on its reproductive and developmental biology mechanisms as well as exploring the nature and role of the rickettsial endosymbionts, found during the course of the study, associated with its reproductive tissues.

Some other previously unstudied aspects of *L. bostrychophila* reproductive biology are also described, including the ultrastructure of the ovaries, egg chorion and egg morphometrics.

1.10. Parthenogenetic reproduction

The term parthenogenesis was first used by Owen (1849), who defined it as the successive production of a procreating individual from a single ovum, without

involvement of any sperm. Parthenogenesis is widespread in plants but in animals is restricted to rotifers, cladocera, and insects (Maynard-Smith, 1978, 1984; Dixon, 1998). Parthenogenesis was known to occur in insects as early as 1745, when Bonnet proved beyond doubt that aphids can propagate without fertilisation and continue to do for so as many as 10 generations.

Parthenogenesis may be classified according to the behaviour of the chromosomes at the maturation division of the oocyte as:

1). *Haplo-diploidy* where a normal division occur in the oocyte, fertilised eggs developing into females, unfertilised eggs into males.

2). *Apomictic (Ameiotic)* parthenogenesis no reduction division occurs so that the offspring have the same genetic constitution as the mother and are all females.

3). *Automictic (meiotic)* parthenogenesis whereby a normal reduction division occurs, but is followed by the fusion of two nuclei (either the female pronucleus with the second polar nucleus, or the fusion of the two cleavage nuclei) so that the diploid number of chromosomes is restored (Fig. 1.3).

Alternatively and more popularly parthenogenesis may be termed according the sex of the offspring produced as

- *Arrhenotoky*: when only males are produced,
- *Thelytoky:* when only females are produced. *Thelotoky* may in turn be facultative or (cyclic e.g. aphids) or obligate (e.g. *L. bostrychophila*).
- *Amphitoky*: whereby individuals of either sex may be produced.

In many cases parthenogenesis has a cytological and genetic base, however in an increasing number of arthropod species it have been shown to be parthenogenic as result of the infection of ovarial tissues by *Rickettsia*-like organisms (RLO).

The high rate of increase of parthenogens and the ability of an individual to establish a colony gives them a considerable advantage over sexual species when colonising new habitats.

Parthenogenesis has been recorded in all insect orders except Odonata, Dermaptera, Neuroptera and Siphonaptera. In the Psocoptera, parthenogenesis is widespread (Ribaga, 1905 ; Monterosso, 1953; Broadhead, 1954; Mockford, 1971) and is either facultative or, more commonly obligatively thelytokous such as in *L. bostrychophila.*

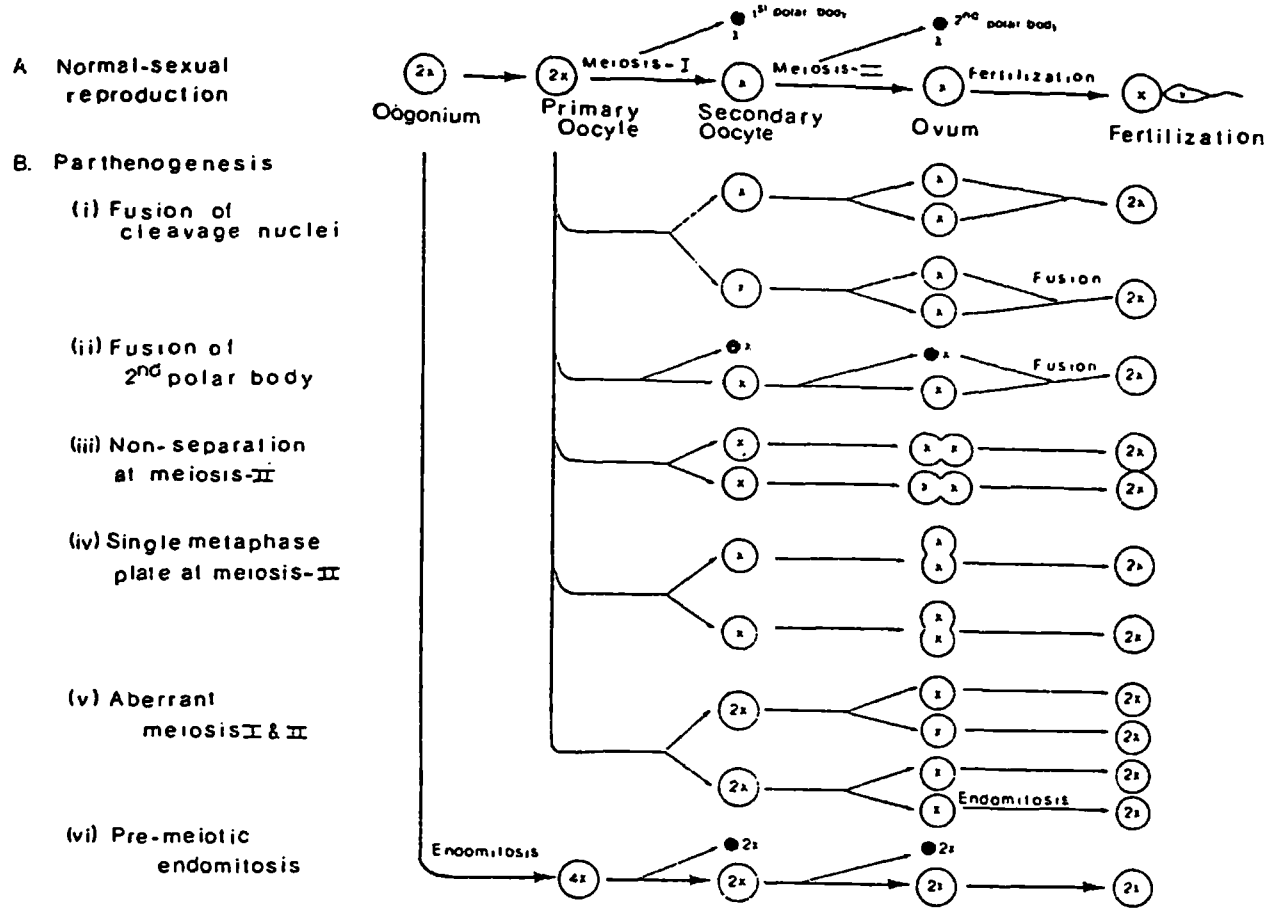


Fig. 1.3. Types of automictic and meiotic parthenogenesis in insects. Data from Kerkut and Gilbert (1985).

Mockford (1971) suggested that thelytoky is widespread in the Psocoptera as it is seen in 13 of the 32 families in the order. However the actual recorded number of parthenogenetic species in total is low (less than 1%), Mockford (1971) quotes a figure of 27 species with confirmed obligate thelytoky and a further 13 species suspected of having similar mode of reproduction.

In the relatively extensively studied aphids species, only 3% are totally parthenogenetic (Blackman, 1980). Most species of aphids reproduce both asexually and sexually, with several generations of apomictic parthenogenesis between each bout of sexual reproduction, i.e. they show cyclical parthenogenesis (Dixon, 1998). The production of sexual morphs (in aphids) is triggered by environmental cues and usually has an annual periodicity (Lees, 1973).

In Psocoptera however, parthenogenesis is for the most part obligate thelytoky (Table 1.2). Only two species of psocids known of having facultative thelytokous forms, *Psocus bipunctatus* (Schneider, 1955) and *Trichadenotecnum alexandrae* (Mockford, 1971), whereby females from the bisexual populations are capable of facultative parthenogenesis. The egg viability of these two facultative parthenogenic psocids is reduced. In some psocids development starts in unfertilised eggs but ceases in embryonic or early nymphal stages. Schneider (1955) observed 14 psocid species in which virgin females laid eggs, in which embryonic development was initiated but ceased at germ layer formation. In the case of *Caecilius piceus* embryonic development went to completion and about half of the eggs hatched, producing weak nymphs. All died in the first instar. The great majority of the parthenogenetic species are either cosmopolitan or temperate species indicating a bias towards the relatively well studied fauna of America and Europe. It is conceivable that many other tropical and subtropical parthenogenetic species are either unknown or that simply there are no data on their reproductive biology.

Table 1.2. Shows the list of families in the Psocoptera with the number of species known to be thelytokous (or probably thelytokous). Among this list ten parthenogenetic species are found in Britain: The cosmopolitan liposcelid *L. bostrychophila* and *L. obscura* as well as *Cerobasis guestfalica* of Atropidae.

In *L. bostrychophila* no males or sperm have never been observed. As a consequence it has a far greater reproductive potential than bisexual liposcelid species colonisation and exploitation of new habitats is therefore enhanced and potential pest status is increased (Turner, 1994).

The phenomenon of parthenogenesis is of interest as an unusual mode of reproduction. The underlying mechanism of the genetics, population biology, reproduction strategy and evolution of parthenogenetic psocids are largely absent in the population genetics literature. The geographical distribution of bisexual and thelytokous species has received very little attention (Mockford, 1971).

Sub-orders	Families	Species	Type of parthenogenesis
1). Trogiomorpha	Lepidopsocidae	*Echmepteryx hageni*	Obligate thelytoky
		Pteroxanium kellogi	Obligate „
	Atropidae	*Lepinotus reticulatus**	Obligate „
		*Cerobasis guestfalica**	Obligate „
	Psyllipsocidae	*Psyllipsocus ramburi**	Obligate/Facultative
2). Troctomorpha	Liposcelididae	*Liposcelis bostrychophila**	Obligate thelytoky
		Liposcelis obscura	Obligate „
3). Psocomorpha	Epipsocidae	*Epipsocus lucifugus**	Obligate „
	Caeciliidae	*Caecilius aurantiacus*	Obligate „
		*C. flavidus**	Obligate „
		C. manteri	Obligate „
	Elipsocidae	*Palmicola solitaria*	Obligate „
		Reuterella helvimacula	Obligate „
		*Elipsocus hyalinus**	Obligate „
		*Pseudopsocus rostocki**	Obligate „
	Psoculidae	*Psoculus neglecta*	Obligate „
	Philotarsidae	*Aaroniella sp.*	Obligate „
		Philotarsus picocornis	Obligate „
	Lachesillidae	*Lachesilla pacifica*	Obligate „
	Peripsocidae	*Peripsocus quadrifasciatus*	Obligate „
		P. stagnivagus	Obligate „
		*P. subfasciatus**	Obligate „
		Ectopsocus meridionalis	Obligate „
		Ectopsocus pumilis	Obligate „
	Psocidae	*Psocus bipunctatus*	Obligate/facultative
		Clematostigma morio	Obligate thelytoky
		Trichadetecnum alexandrae	Obligate facultative

Table 1.2. The known prevalence of parthenogenesis in the three sub-orders of the Psocoptera. Data from Mockford (1971). * Found in the UK

Badonnel (1951) observed that the males of the psocid *Reuterella helvimacula* of the family Elipsocidae and *Cerobasis guestfalica* in Europe are found primarily in the northern parts of the range of these species. Thornton and Wong (1968) noted that the males of *Ectopsocus pumilis* (Peripsocidae) are only known from southern India. Smithers (1969) also reported that males of *Elipsocus hyalinus* (Elipsocidae) were found

in Scotland. However elsewhere in Britain only females of this species have been found (Kimmins, 1941; Thornton and Broadhead, 1954).

The results of a study carried out on the geographical distribution of the sexes of five common psocids in the eastern United States by Mockford (1971) have shown striking similarities. The five psocid species studied here were *Echmepteryx hageni, Caecilius aurantiacus, Peripsocus quadrifasciatus, Trichadenotecnum alexandrae* and *Psocidus pollutus*. The first four are capable of thelytoky, while the fifth is presumed to be thelytokous in the parts of its range where males are unknown. Mockford (1971) reasoned that bisexual aggregates of these must, in general, be older than the thelytokous ones, which must be derived from them. The author proposed that the biased distribution was due to either replacement of wide-ranging bisexual forms by the thelytokous forms or the spread of thelytokous forms having refugia inhabited by the bisexual forms. Some obligate thelytokous species which obviously are the derivatives of the bisexual species have not yet been distinguished nomenclaturally from their bisexual parental forms. In many cases an aggregate of parthenogenetic and bisexual forms having no known morphological differences were maintained as single specific name whilst others with striking morphological similarities were arranged in species complexes or sub-groups (Mockford 1971; Schneider, 1955).

1.11. Psocid and *Rickettsia*-like organisms endosymbiotic complex

During the electron microscopical examination of the reproductive organs in this study, intracellular bacteria were found. This situation is not uncommon in the Insecta. There are approximately 250 identified species of bacteria which have been associated, in one way or another, with insects and ticks (Steinhaus, 1947). This does not include the intracellular forms such as the rickettsiae and other intracellular "symbionts". Many insects live in obligate symbiosis with mutualistic fungi and bacteria (Norris, 1972). These types of symbiosis were first recognised by Witlaczil (1884). All major insect orders are known to be closely associated with microrganisms. Symbiosis is used here to mean close association between insects and microrganisms. This association may vary from mutualistic, commensal to downright parasitism (Ahmadjian and Pracer, 1986;

Goff, 1985; Boucher, 1982, 1985; Margulis, 1981; Starr, 1975; Schmidt and Roberts, 1985; Ishikawa, 1989 and Douglas, 1989, 1992).

The term 'endosymbiont' is also adopted throughout this study to refer to the close association between a group of *Rickettsia*-like bacteria and its host insect, regardless of their trophic role. The term *aposymbiont* is also used to refer to those insects experimentally rendered *Rickettsia*-free.

The experimental demonstration of specific interdependencies among species in such symbiotic complexes has only occurred relatively recently (e.g., Norris and Baker, 1967; Norris *et.al.*, 1969; Abrahamson and Norris, 1970; Chu *et.al.*, 1970; Kok *et.al.*, 1970).

Many insects living on a deficient diet are known to have specialised symbiont containing cells called mycetocytes. The mycetocytes are organised in specialised tissues called mycetomes. The mycetomes are located in different organs of the insect body, although in the majority of cases, the function of the symbionts is either imperfectly known or unknown. The relationship is logically considered as largely symbiotic because of its very intimate nature (Pant, 1972).

Insects with a restricted diet, deficient in certain essential nutrients, like the sap-sucking aphids (Adams, 1996; Douglas, 1989, 1994), wood inhabiting termites (Radek *et.al.*, 1999; Belitz *et.al.*, 1998) and those blood-sucking insects which never feed on anything except blood (*Glossina* sp, *Aedes*, Ruduviids, Anoplura etc.) are all known to have mycetocytes (Burgdorfer *et.al.*, 1973; Kokwaro *et.al.*, 1990).

Those blood-sucking which at some stage partake of other food like fleas, blood-sucking Nematocera or Tabanidae with free living larvae do not house microrganisms (Wigglesworth, 1929). This underlines the nutritional role of these symbionts.

Successful removal of endosymbiotic bacteria using antibiotics in some insects that feed on deficient diet can cause deleterious affects (Brooks and Richards, 1955; Brooks, 1970). Experiments on the cockroach *Blatella germanica* and some stored product beetles - *Lasioderma serricorne, Stegobium panaceum, Oryzaephilus* sp. and *Sitophilus* sp., in which aposymbiosis was produced, gave rise to weak nymphs and growth was arrested (Brooks and Kringen, 1972; Norris, 1972; Pant, 1972).

The condition could be reversed if aposymbiotic insects were provided with a supplemented artificial diet containing vital vitamins, amino acids and cholesterol (Pant, 1972).

Douglas (1998) produced the most recent review of the interaction in insect-microbial symbiosis, giving special emphasis on aphid-endosymbionts system. The symbiosis in aphid bacteria has a nutritional basis. Bacteria-free aphids grow poorly and produce few or no offspring.

Not all insect-bacterial associations can be explained in the light of bacteria providing additional nutrients. *L. bostrychophila* for instance is fed a very balanced diet consisting of powdered milk, yeast, wholemeal flour and dried milk; nevertheless all individuals are infected regardless.

Many species of bacteria have been identified as insect pathogens. Their pathogenicity to insects is usually due to the production of host specific toxins. The best known of these is *Bacillus thuringiensis* (BT), a spore-forming bacterium, pathogenic to nearly 200 species of pest insects, particularly Lepidoptera. BT has been developed as one of the major agents used in the biological control of insect pests. *Bacillus larvae*, another member of the same genus, causes American foulbrood, a serious disease of honeybee larvae (Krieg, 1987). In addition to the members of the genus *Bacillus*, certain species of *Clostridium, Pseudomonas, Serratia, Streptococcus* are also insect pathogens (Faust, 1974; Krieg, 1987). Many bacterial diseases of plants and humans are mechanically transmitted by insects; for example, those that cause plague (*Yersinia pestis*) in humans. Other bacteria lacking cell wall, like the Spiroplasmas (agent of the yellow disease and witches broom diseases of plants) and Mycoplasmas are found in a number of insect groups (Krieg, 1987; Tiivel, 1989).

The most commonly occurring microrganisms in insects, however, are the *Rickettsia*-like organisms. They are found in Blattodea, Isoptera, Homoptera, Heteroptera, Anoplura, Mallophaga, Coleoptera, Hymenoptera and Diptera. In the Psocoptera, early papers of (Sikora, 1918) and Hertig and Wolbach (1924) described *Rickettsia*-like organisms from a wide range of insect species including two different psocopterans. Subsequent workers on psocid anatomy, however, (Badonnel, 1934; Finlayson, 1949; Goss, 1952, 1954; Jostes, 1975; Seeger, 1979; Wong, 1970; Wong and Thornton, 1966; Wong and

Thornton, 1968) have failed to find, mention or identify any microrganisms in the bodies, reproductive organs or eggs of psocids they examined. To our knowledge this study remains the first of its kind undertaken in the order Psocoptera dealing with endosymbiotic rickettsiae.

Some rickettsiae are insect pathogens, and are normally very slow to kill (parasitism). The rickettsiae comprise a collection of bacteria that share the property of an intimate association with eukaryotic cells (Girin and Buoletreau, 1995). *Rickettsia* and some related genera are obligatory intracellular bacteria. Several genera (*Rickettsia, Anaplasma, Cowdria, Ehrlichia*) are transmitted by arthropods and cause disease in mammals (Werren *et.al.*, 1994).

The order *Rickettsiales* contains three families (*Rickettsiaceae*) (Weisburg *et.al.*, 1989). These three families are:

A) Typhus Group Rickettsiae: *Rickettsia typhi* (the agent of Murine Typhus), *Rickettsia rickettsii* (Rocky mountain spotted fever) *R. tsutsugamushi* (scrub typhus), *R. prowazekii* (Epidemic Typhus), *R. canada.*

B) *Ehrlichieae* Group Rickettsiae: *Ehrlichia canis, E. sennetsu, E. risticii, E. phagocytophila, E. equi.*

C) *Wolbachieae* Group Rickettsiae: containing *Wolbachia pipientis* (insect endosymbiont), *W. persica* (not associated with a known disease of humans or animals).

In the rickettsiales a rigorous set of criteria of classification have been applied to define the species. However the degree of relatedness among these microrganisms in some cases remains unclear (Braig *et.al.*, 1994; Coyne, 1992).

The principal genus affecting invertebrates is *Wolbachia*. Bacteria in the genus *Wolbachia* are cytoplasmically inherited rickettsiae that are found in reproductive tissues (ovaries and testis) of a wide range of arthropods (O'Neill *et.al.*, 1992; Rousset *et.al.*, 1992; Stouthammer *et.al.*, 1993; Werren and Jaenike, 1995; Werren *et.al.*, 1995). The presence of *Rickettsia*-like microrganisms was first discovered in the gonads, and apparently in no other organ in the mosquito *Culex pipiens* (Hertig and Wolbach, 1924).

Subsequently the name *Wolbachia pipientis* was proposed for the *Rickettsia* (Hertig, 1936). *Wolbachia pipientis* was later recognised as the species type for the genus (Werren, 1997).

Recently there has been an upsurge in the research interest of these parasitic bacteria. They are known to cause a number of reproduction alterations in their hosts, including cytoplasmic incompatibility between strains (Clancy and Hoffmann, 1996; O'Neill and Karr, 1990) and related species (Breeuwer *et.al.*, 1992; Breeuwer and Werren, 1990), parthenogenesis inducing (PI) (Stouthamer, 1993) and feminisation of genetic males (Rousset *et.al.*, 1992).

1.12. Distribution of *Wolbachia* in the Arthropoda

Wolbachia are extremely widespread (Table 1.3.). A recent survey of neotropical arthropods (Werren *et.al.*, 1995) found these bacteria in over 16% of insect species, including each of the major insect orders, Coleoptera, Diptera, Hemiptera/Homoptera, Hymenoptera and Orthoptera. Surveys of insects from temperate, tropical and other geographical regions are lacking, but a similar percentage of infected species can be expected (Werren and Winsor, unpublished). Nevertheless, extrapolating from the percentage of infected species to the estimated number of species known to exist globally (10-30 million), Werren, 1997 calculated 1.5-5.0 million infected insect species world-wide, thus making *Wolbachia* among the most abundant of parasitic bacteria.

However there are little data on the actual distribution among the arthropods. The only systematic survey of *Wolbachia* distribution was carried out in neotropical arthropods (Werren, *et.al.*, 1995). This study only covers some species in eight insect orders, and no psocopteran was examined, although psocids both wingless and free-living species are readily available in many neotropical habitats.

Wolbachia is also widespread in terrestrial isopods and has been found in 17 species (Juchault, *et.al.*, 1994; Rousset, *et.al.*, 1992). A feminising strain of *Wolbachia* is found in the isopod *Armadillidium vulgare*, whereby genetic males are transformed into competent females (Rousset *et.al.*, 1992).

Insects	
Order	
Diptera	Mosquitoes *Culex* sp. *(Wolbachia pipientis)*
	Aedes sp.
	Fruitflies *Drosophila* sp.
	Blowfly *Calliphora* sp.
Hymenoptera	Parasitic wasps e.g. *Nasonia*
Coleoptera	Flour beetles *Tribolium*
	Alfalfa Weevil
	Ladybird *Coccinellidae*
Lepidoptera	Flour moth *Ephestia*
Homoptera	Planthoppers
Other groups	
Isopoda	Woodlice *Armadillidium*
Acarina	Mites Citrus and Spider mites
Nematodes	Filarial worms

Table 1.3. Distribution of *Wolbachia* in the Arthropoda. Data extracted from Werren (1997).

There are also known cases of *Wolbachia* occurring in mites (Johanowicz and Hoy, 1995) and filarial worms (Sironi *et.al.*, 1995). More interesting however, the feminising strain found in the isopod *Armadillidium vulgare* and that of the filarial worm are closely related to those found in insects (O'Neill *et.al.*, 1992), indicating both the possible horizontal transmission between insects and isopods as well as the extension of the distribution of *Wolbachia* to other phyla (Werren, 1997).

The phenomenon of parthenogenesis is of interest as an unusual mode of reproduction. The underlying mechanism of the genetics, population biology, reproduction strategy and evolution of parthenogenetic psocids are largely absent in the population genetics literature.

The ultrastructure and the role of *Wolbachia*-like microrganisms in *L. bostrychophila* are considered in chapter three. Experiments dealing with decontamination and the production of aposymbiotic individuals using heat and antibiotic treatment are summarised in chapter four. A molecular approach adopted to characterise and gene sequence the endosymbiotic bacteria is covered in chapter five. Finally the selective implications of this interoocytic bacteria on the reproduction output of parthenogenetic *Liposcelis bostrychophila* populations, in terms of population fitness and survivorship is ɔlored in the penultimate chapter.

CHAPTER TWO

GROSS MORPHOLOGY AND ULTRASTRUCTURE OF THE INTERNAL REPRODUCTIVE SYSTEM AND EGGS OF *L. BOSTRYCHOPHILA*

CHAPTER TWO

2.0. GROSS MORPHOLOGY AND ULTRASTRUCTURE OF THE INTERNAL REPRODUCTIVE SYSTEM AND EGGS OF *L. BOSTRYCHOPHILA*

2.1. INTRODUCTION

Psocids have been traditionally identified using morphological characters and relatively little attention has been paid to their anatomy. The early accounts of psocid anatomy were given by Badonnel (1934). The internal anatomy of *Psyllipsocus ramburi, Ectopsocus meridionalis, Stenopsocus stigmaticus, Metylophorus nebulosus* were investigated by Badonnel (1949). He also provided descriptions of the reproductive, nervous, digestive and musculature systems. All these are however from winged species.

Knowledge of the internal structures of the wingless genus *Liposcelis* is restricted to the work of Goss (1953, 1954) on the reproductive organs and embryology. Goss's (1954) paper on the ovarian development and oogenesis in the booklouse, *Liposcelis divergens (?bostrychophila)* represent the only attempt to study the internal structures in the genus *Liposcelis*. Goss (1953) provided a description of the gross morphology of the reproductive system of this species.

The process of oogenesis has been described by Mahowold (1972) as the central preparatory event for embryogenesis, and is considered to be a series of biological functions whereby the egg cell is endowed both with the nutritional prerequisites for normal development and with the necessary information content so that proper differentiation occurs.

Most recently Buning (1994) reviewed the insect ovary giving special emphasis to its ultrastructure, previtellogenic growth and evolution. The types of oogenesis vary greatly from one group of insects to another depending the type of ovariole development.

There has been only one ultrastructural study of a psocid's reproductive systems, that of Buning and Sohst (1990) on the ovaries *Peripsocus phaeopterus* and *Stenopsocus stigmaticus*. Wearing-Wilde (1995) studied the sclerotised spermatophore of the barklouse *Lepinotus patruelis* Pearman.

Insect ovaries develop from mesodermal tissue, which shelters germ cells and their derivatives, the oocyte and eventually the nurse cells (Buning, 1994).

The ovaries of liposcelids are simple tubes in which one egg matures at any given time (Wong and Thornton, 1968). The ovariole type of *L. bostrychophila* is defined as polytrophic meroistic, in which nutritive cells migrate into the vitellarium and alternate with oocytes, the whole being enveloped by a sheath of follicular cells. Polytrophic ovaries are characterised by a follicle consisting of one oocyte together with a well-defined number of trophocytes or nurse cells and the whole complex being surrounded by a sheath of follicle cells. Oogonial differentiation takes place in the germarium. Oocyte differentiation in this type of ovariole has been extensively studied in *Drosophila* by King (1970); King and Buning (1985).

Panoistic ovarioles on the other hand have their germarium merely containing the oogonia (primary oocyte) and mesodermal prefollicular tissue. The follicle is the only trophic tissue. This type of ovariole occurs in the Apterygota, Odonata, Orthoptera and in some Coleoptera.

The process of oogenesis in polytrophic ovarioles is markedly different from that of panoistic ovaries. In polytrophic ovaries after the 16 cell cyst has become surrounded by follicle cells, all the chromatin becomes clumped in one region called the karyosome (Wheeler, 1996). The dimension of the karyosome gradually increases during the subsequent growth stages of the oocyte until when the chromatin material is spread out over the whole nucleus (Koch *et.al.*, 1967).

Details of egg structure in psocids are poorly understood. In Hinton's (1981) comprehensive study on insect eggs, no psocopteran eggs were described. Pearman (1927, 1928) carried out some measurements of the eggs of *Pteroxanium squamosum* Enderlein and members of the family Atropidae (Pearman, 1923). New's (1969 a, b; 1971) studies on the life history and biology of bark-dwelling species include some biological observations on egg mortality, oviposition, eclosion, ecdysis and nymphal stages. A very brief description of the eggs of *L. divinatoria (= bostrychophila?)* was given by Pearman (1928). Some observations on the eggs of the various species of *Liposcelis* were noted by (Broadhead and Hobby, 1944a).

The central theme of this chapter is to describe the gross morphology and the ultrastructural details of the reproductive system of *L. bostrychophila* and two closely related bisexual psocid species Some aspects of the ultrastructure of their polytrophic ovaries and oogenesis are also examined. The ultrastructural changes in a maturing oocyte, will be used as evidence which allows a functional interpretation of the structures. In addition to a range of smaller organ systems, the abdomen of this species also contains the bulk of the digestive system. Spatial relationships between reproductive and digestive systems in the abdomen are also illustrated. Developmental stages of the ovary and oogenesis a process leading to the production of a mature egg in this insect species, are illustrated using light and electron microscopy.

2.2.1. PSOCID STOCK ACQUISITION AND MASS REARING

From Nov. '95 until mid 1997 a series of 30 cultures of *L. bostrychophila* was established from individuals acquired from psocid related consumer complaints or samples obtained from surveys of flourmill premises. Table A2.1 (Appendix), shows the geographical origin of these populations.

Egg output and adult mortality were used as indicators of fitness between these different populations of *L. bostrychophila*. They were also the source of the infected insect tissues used for staining, EM and molecular work.

Liposcelis bostrychophila cultures were routinely fed a diet comprising a mixture of wheat germ, wholemeal flour, powdered milk and yeast in equal proportion in terms of volume (Turner and Maude-Roxby, 1988). Psocids were kept in glass bottles 6cm length x 2.5cm diameter, part filled with the diet. The inner surface of the rim of these glass bottles was painted with 'Fluon'™, a slippery material to prevent psocids from escaping.

Small pieces of folded standard tissue paper (Whatman™) were used as ovipositing substrates. The paper was "concertina-ed" as *L. bostrychophila* prefers to lay eggs along the ridges.

The glass bottles were kept in individual plastic boxes (10x11x7cm), with tight-fitting lids. Relative humidity is a crucial environmental factor in rearing both nymphs and adults of *L. bostrychophila*. The favourable rh% ranges from 65-85% which together with food and temperature determines the number of eggs laid by any population (Turner, 1994). Humidity was maintained at 75% rh in the plastic boxes using a saturated NaCl solution. The stock populations were kept in identical conditions at room temperature (20-25°C).

2.3. MATERIAL AND METHODS

2.3.1. Preparation of *L. bostrychophila* for E.M. examination

Preparations and processing the psocid material, especially embedding and sectioning was extremely difficult. Wax embedded psocid material did not give satisfactory results because of the small size of the insect and the lack of support provided by the wax during ultrathin sectioning. Spurr's resin (Spurr, 1969) embedded psocid materials were more successful and used for the ultrathin sections.

The resin sections tended to fall off from the grids. A Formvar coating, applied to prevent sections from being dislodged, created a thin layer on top of the section and reduced the resolution. Other techniques employed to overcome some of these difficulties included decapitation, and placing psocids in a vacuum to force the fixatives and resin into the abdominal organs.

Live adult psocids of *L. bostrychophila* were placed in 2.5% gluteraldehyde in 0.1M sodium cocodylate buffer (pH 7.7) and then decapitated. Samples were then put under a light vacuum for approximately 12 hours to assist the penetration of the fixative for improved resolution with electron microscope.

To improve contrast, samples were post-fixed with a 1% solution of osmium tetroxide after fixation prior to embedding and sectioning. Samples were dehydrated through an acetone series. Gradually increasing proportions of Spurr™ resin (Spurr, 1969) were used for infiltration until the acetone was completely replaced with 100% resin. Samples were left on a rotator overnight to enhance infiltration, embedded in moulds and transferred to an oven at 60°C for two days to polymerise the resin.

'Silver' sections (60-80nm) were cut from the resin embedded psocid samples, by means of glass knifes using a Reichert ultramicrotome and mounted on Formvar coated grids. Diamond knives were occasionally used to aid sectioning of the cuticle of the body surface (tough and brittle) and to reduce marking the sections, which may occur using glass knives.

Grids were post-stained in 2% Uranyl Acetate followed by Reynold's lead citrate. Grids were then viewed in a Jeol 100 CX MII transmission microscope.

2.3.2. Serial sections: *L. bostrychophila* were fixed, embedded and cut as described in the previous section. Sections, aligned on the insect's longitudinal axis, were 1μ thick and taken at 5μ intervals. These sections were then stained with Toluidine Blue and heat dried for 10 seconds directly on a slide. Sections were then photographed using camera (C35 AD-2) mounted on Olympus microscope (Vanox-T). Images from the serial sections were used to reconstruct the structure of the internal abdominal anatomy of *L. bostrychophila.*

2.3.3. Egg Morphometrics

Variability in egg size and weight were compared between 10 of the *L. bostrychophila* populations. In addition the ratios of egg size to adult size were compared with other published results.

1. ***Egg Wet And Dry Weight***: Eggs were washed in distilled water, to remove any impurities/detritus attached on their surface, and placed in a tin foil. Twenty eggs per Populations were then dried in an oven at 70°C to constant weight. Weighing (to 0.001 mg) was carried out using a Cahn electrobalance 4400.
2. ***Egg sizes***: Egg dimension (Max. width x Max. length) of the populations (20 eggs of each population) of *L. bostrychophila* were measured using an eyepiece micrometer (*1micrometer division = 0.057mm).*

2.4. RESULTS

2.4.1 Gross anatomy and the architecture of *L. bostrychophila* ovaries

The gross morphology and histology of the reproductive and digestive systems of *L. bostrychophila* were reconstructed from the ultrathin longitudinal serial sections (Fig. 2.1).

The digestive system of this species is relatively short. The 'crop' wall thickens to approximately 75μm at the anterior end where the oesophagus joins. Serial sections have shown that the thickening is due to the proliferation of secretory microplicae. These cells are mononucleate, cylindrical and highly orientated, attached to the gut basement membrane (Fig. 2.2).

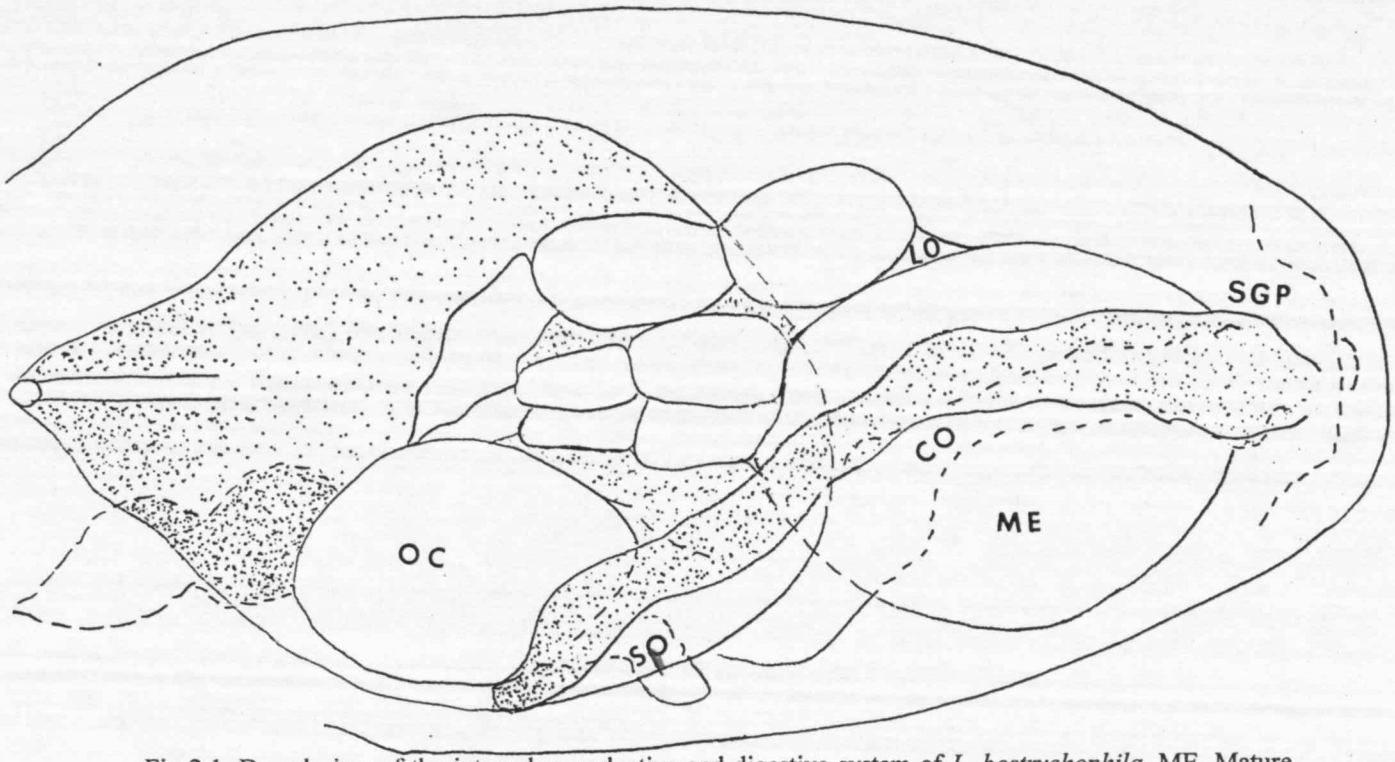


Fig 2.1. Dorsal view of the internal reproductive and digestive system of *L. bostrychophila.* ME, Mature Egg; OC, Oocyte; LO, Lateral Oviduct; CO, Common Oviduct; SP, Spermatheca; SGP, Subgenital Plate.

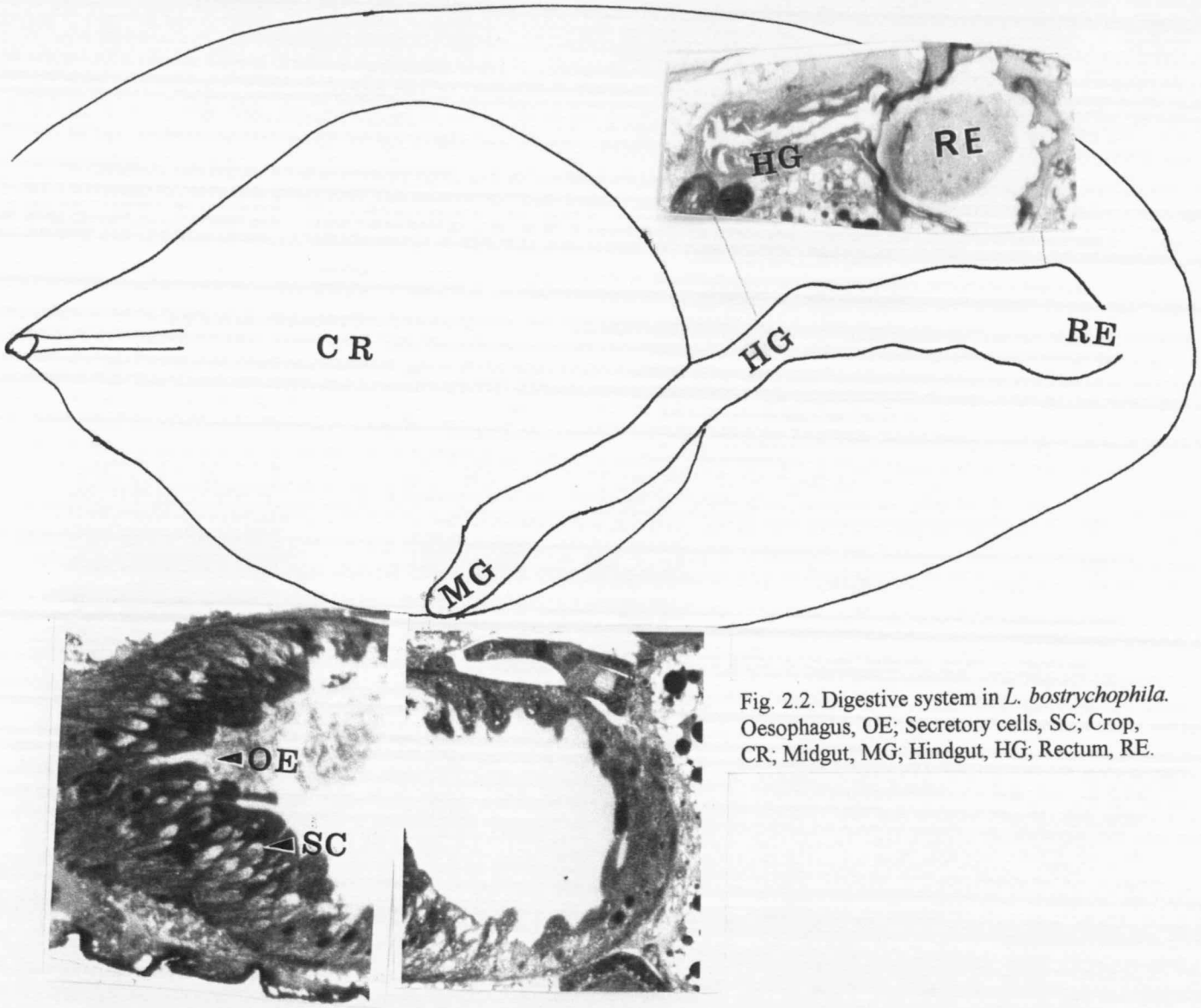


Fig. 2.2. Digestive system in *L. bostrychophila*. Oesophagus, OE; Secretory cells, SC; Crop, CR; Midgut, MG; Hindgut, HG; Rectum, RE.

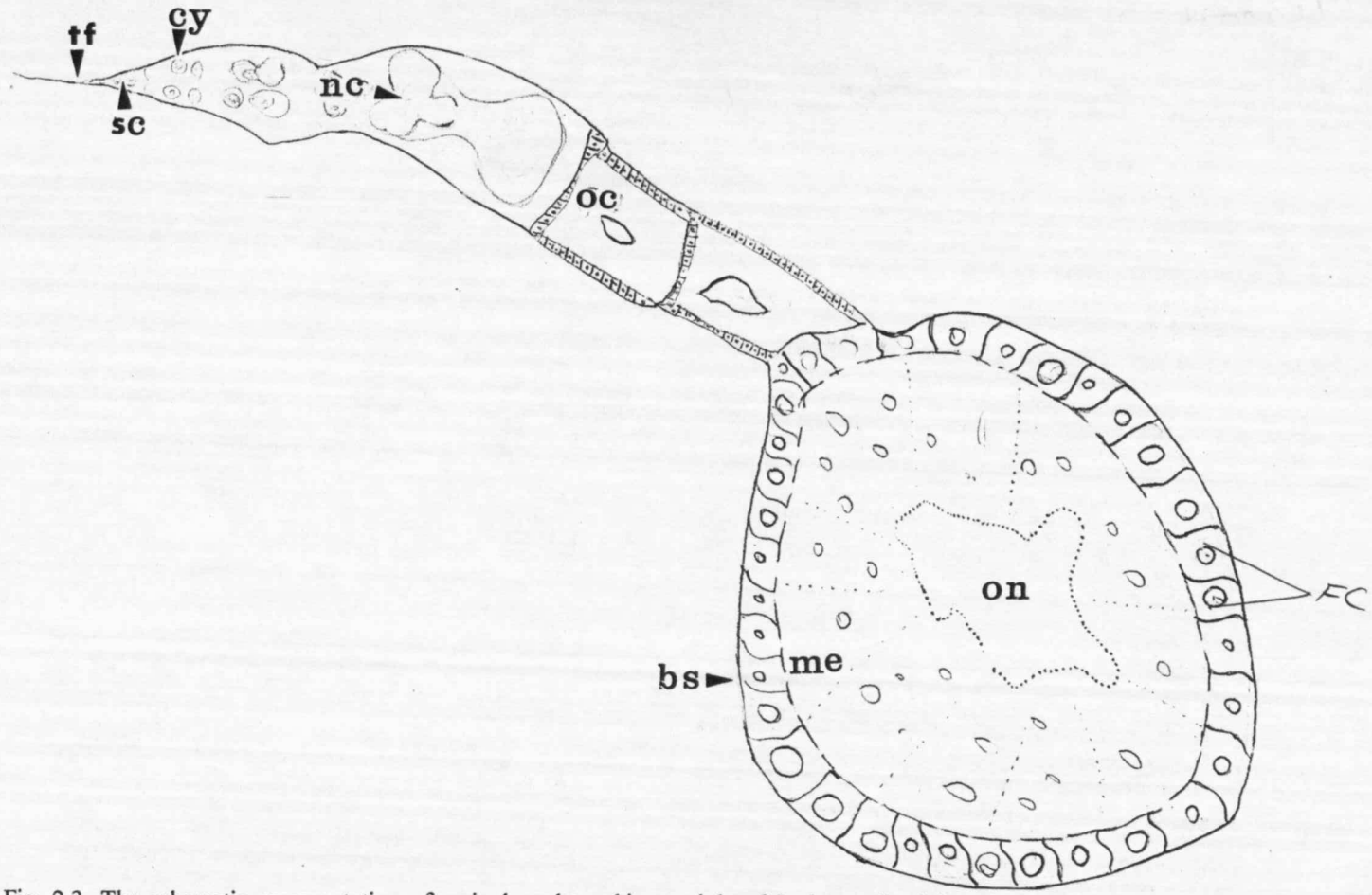


Fig. 2.3. The schematic representation of a single polytrophic ovariole of *L. bostrychophila*. The three zones of the ovariole are indicated. The ovariole arrangement in *L. bostrychophila* represent the basic structure of polytrophic meroistic type ovaries in insects. TF, Terminal Filament; SC, Stem Cells; Cy, Cystocytes, NC, Nurse Cells; OC, Oocyte; ME, Mature egg; BS, Basement Membrane; ON, Oocyte nucleus; FC, Follicle Cells;

The cells lining the dorsal, ventral and the posterior regions of the 'crop' are less prolific and short, 8μm long, while those at the anterior end are much longer (65μm) and it is through these that the lumen (30μm in diameter) from the oesophagus passes. This thick foregut is followed by a short midgut and the hindgut, together approximately 0.4mm in length.

The psocid ovaries each consist of 5 tubular ovarioles, which contain the developing oocytes arranged in a linear fashion. Each ovariole is composed of an outer membrane referred to as tunica externa (also as tunica propria or the basement membrane). This acellular membrane is attached to the front of the terminal filament, the germarium and all the developing egg chambers. The posterior part of the germarium and the vitellarium is covered by a tubular epithelial sheath of, occasionally binuleate, follicular cells (Fig. 2.3). This outer envelope (basement membrane) bears numerous trachaeoles and microtubules of the attached muscles, which move the ovariole to discharge the egg during oviposition (Plate 2.1).

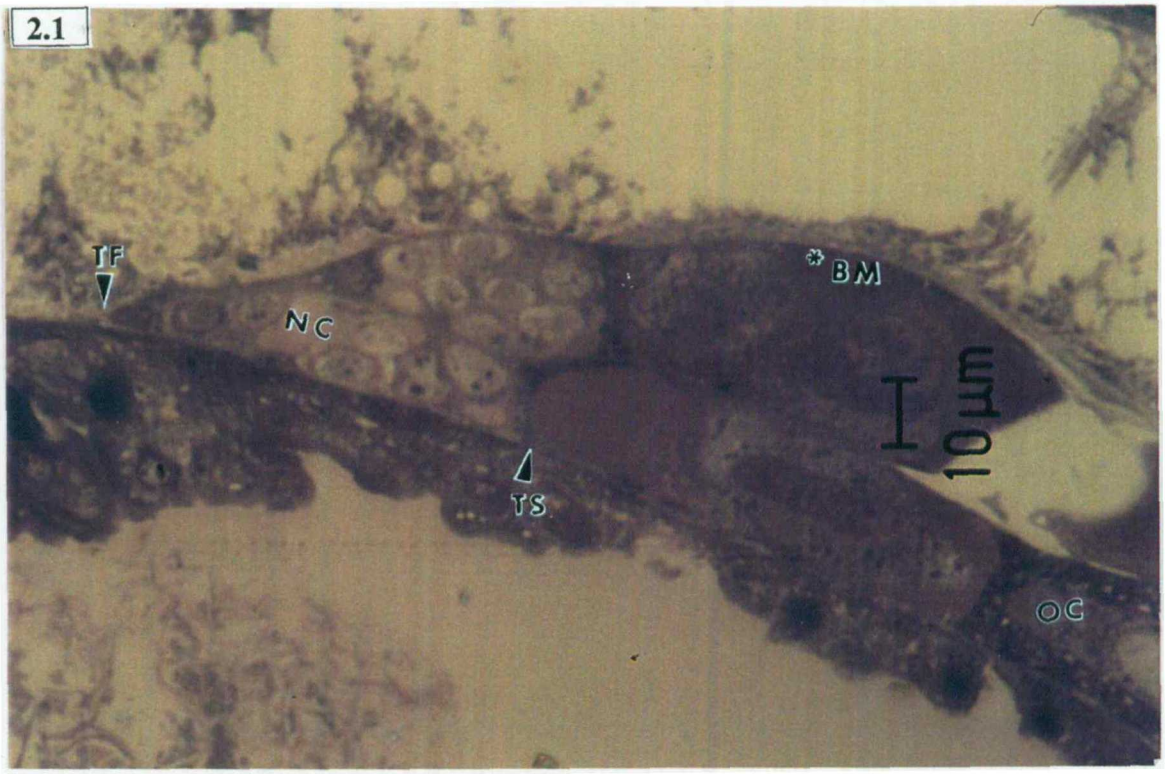


Plate 2.1. Ultrathin section of the germarium of *L. bostrychophila* delineated by a thick basement membrane and divided by transverse septa. TF, Terminal filament; TS, Transverse Septum; BM, Basement Membrane; NS, Nurse Cells, OC, Oocyte.

The structure of the ovariole varies according to the degree of development of the oocyte within. The ovarioles are differentiated into a terminal filament, an anterior sausage shaped germanium and posterior vitellarium. Germarium and vitellarium constitute the portion of the ovariole known as the egg tube.

At their anterior, or distal ends, the ovarioles of *L. bostrychophila* are drawn out into thin terminal filaments which join one another to form a suspensory ligament which is attached to the dorsal abdominal diaphragm in the second abdominal segment. Plate 2.1, shows the structure of the anterior portion of *L. bostrychophila* ovarioles, the germarium. The germarial region of the adult *L. bostrychophila* is short measuring 23μm. The basement membrane of this psocid species bifurcates between the end of the germarium and beginning of the terminal filament. In thin sections the basement membrane of two adjacent ovarioles appear as a honeycomb structure. Plate 2.1, shows the tip of germarium as an elongate, multicelled and slightly curved structure. The morphological differentiation between the nurse cells and the oocyte in the lower part of the germarium become more marked. Each ovariole narrows to a stalk, or pedicel, which extends posteriorly and unites with others to form a very short lateral oviduct. These lateral oviducts, one from each ovary of five ovarioles, fuse together to form a single tubular structure called the common or median oviduct (Fig. 2.1), which extends to the outside at the distal rim of the seventh abdominal segment. This (common oviduct) opens into a pouch or tube of the eighth abdominal segment called the genital chamber. The genital chamber of *L. bostrychophila* functions only as the pathway for ripe eggs as there are no copulatory activities involved. *Liposcelis bostrychophila* appears to have a fully developed but not functional spermatheca (Fig. 2.1), inserted directly into the lower end of the common oviduct. Following copulation in bisexual species, sperm are stored and nourished in this organ until used to inseminate the ripe eggs via the micropyle. The presence of the non-functioning spermatheca and the micropyle are indications of the sexual origin of what is now a completely parthenogenetic species. The genital chamber opens to the exterior via the gonopore towards the rear end of the eight abdominal sternite, the sub-genital plate. The gonopore is associated with two gonapophyses at either side. The subgenital plate lies beneath the two lateral gonapophysis and has a characteristically T-shaped sclerite in the genus *Liposcelis* and appears as a region of

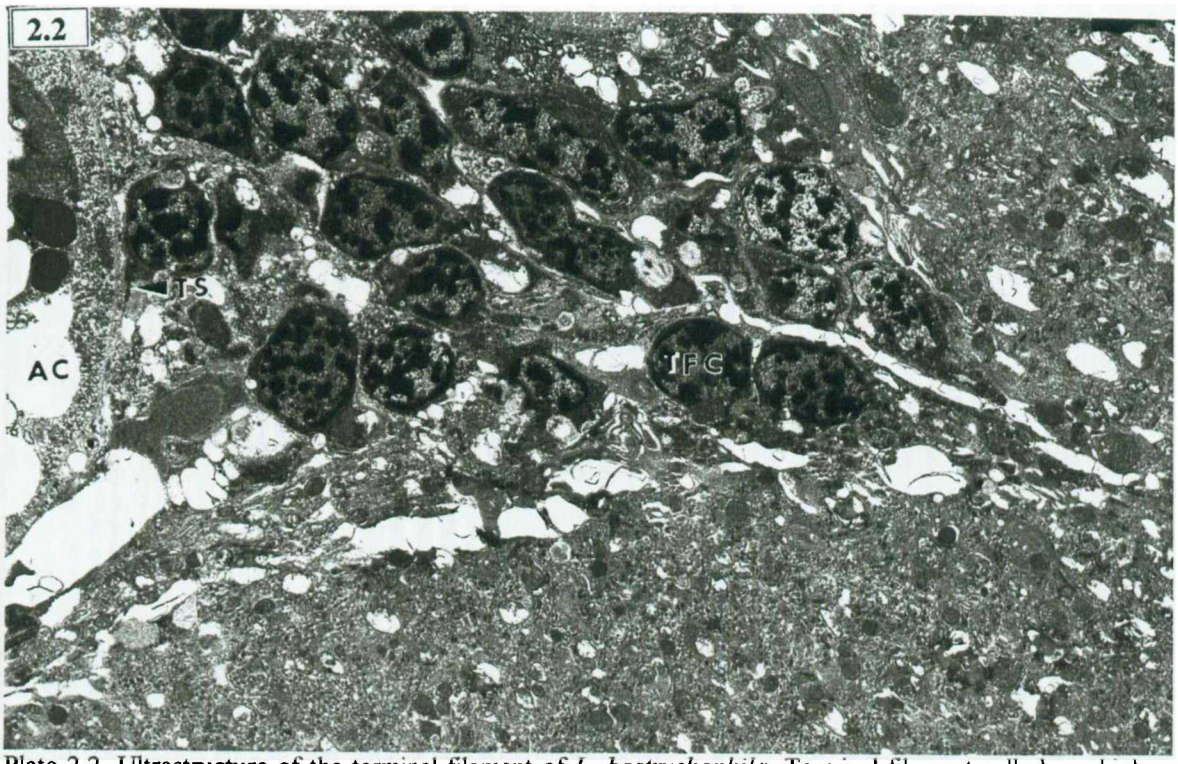


Plate 2.2. Ultrastructure of the terminal filament of *L. bostrychophila*. Terminal filament cells have high electron density and cytoplasm with high nucleus/cytoplasm ratio TFC, Terminal Filament Cell, TS, Transverse Septum, AC , Apical Chamber. Magnification 5800x.

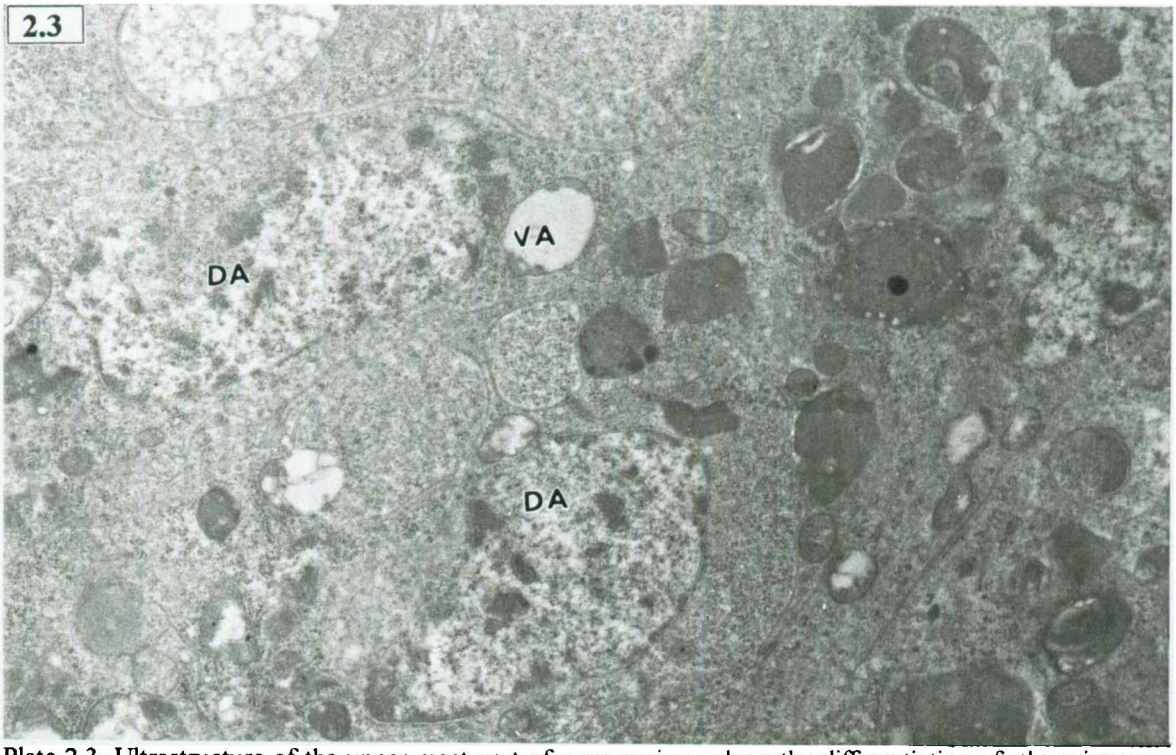


Plate 2.3. Ultrastructure of the upper most part of a germarium where the differentiation of the primary oogonium into daughter cells occurs. DA, Daughter Cells Nuclei; VA, Vacuoles. Magnification 14400x.

thickened cuticle in the serial sections.

2.4.2. Ultrastructure of the ovariole

Polytrophic ovarioles of psocids can readily be divided into five regions on the basis of their general morphology (Fig. 2.3). At the anterior end of the ovariole lies the terminal filament, composed of flattened cells, which attaches the ovarioles to the thoracic wall. The germarium comprises the second region with anterior portion containing one or more stem line oogonia and various daughter cells. These cells subsequently differentiate into an oocyte and the rest into nurse cells. In the central region of the germarium, pre-follicular cells grow between clusters of cells, and finally in the posterior region of the typical egg chamber is formed, with oocyte located posterior to the nurse cells and the nurse cells-oocyte complex surrounded by a single cell thick follicular epithelium.

2.4.3 The terminal filament

The terminal filament of the ovary of *L. bostrychophila* consists of thin elongated somatic cells, with highly dense cytoplasm. These cells bear numerous projections that are predominantly orientated parallel to the long axis of the ovarioles. In this species a transverse septum (membrane) separates the terminal filament from the germarium lying ventral to it (Plate 2.2). The terminal filament cells measure 1.14 ±0.26 μm (mean diameter ± 1 SD). Terminal filament cells have high density cytoplasm, with high nucleus/cytoplasm ratio.

2.4.4 The Germarium

Below the terminal filament lies the germarium (Plate 2.3). This contains the oogonia, enveloped in a layer of mesodermal cells. The primary oogonium divides synchronously into daughter cells (cystoblasts) some of which become the definitive oogonia whilst the rest develop into follicle-nurse cell complex (Plate 2.4). Oocytes pass down the ovariole into the vitellarium enlarged and ensheathed by the prefollicular tissues. In the central region of the germarium, prefollicular cells grow between clusters of cells, and finally in the posterior region the typical egg chamber is formed, with the oocyte located posterior to the nurse cells and the nurse cell-oocyte complex (Plate 2.4).

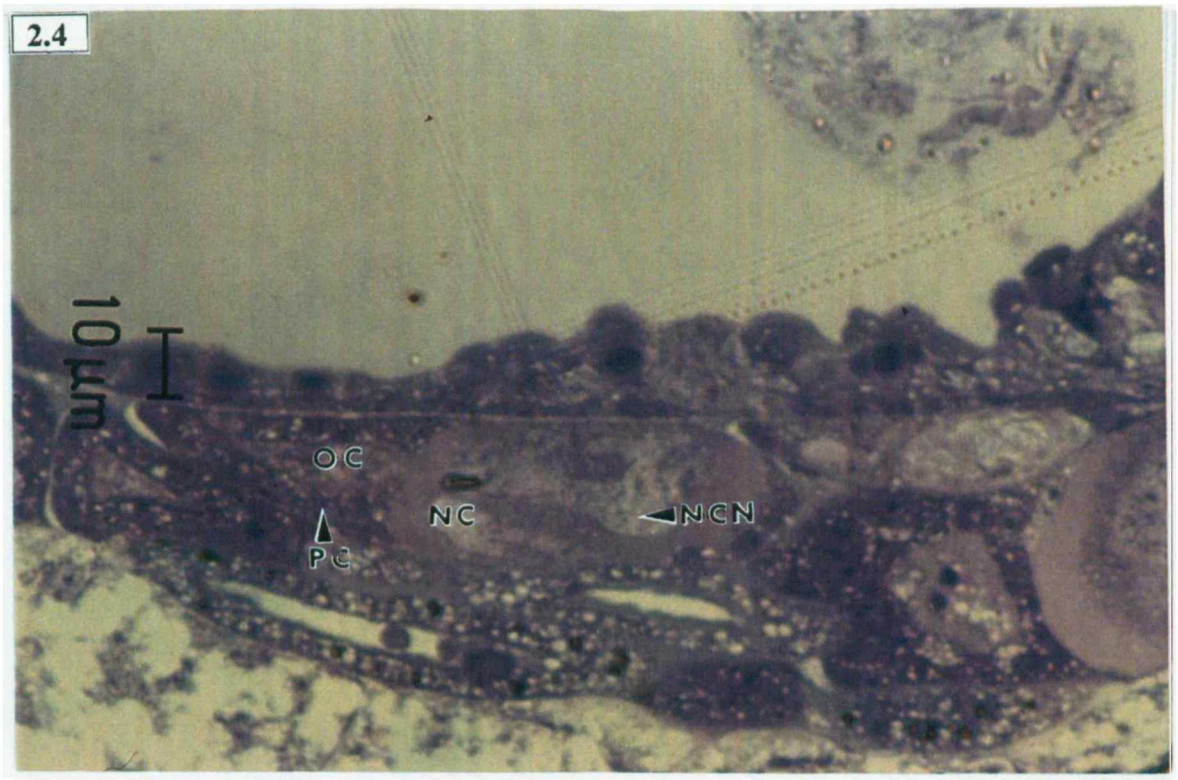


Plate 2.4. Ultrathin section of the central region of the germarium showing the formation of nurse cell-oocyte complex. NC, Nurse Cells; NCN, Nurse Cells Nuclei; PC, Prefollicular Cells. OC, Oocyte Chamber.

2.4.5 The nurse cells

The nurse cells, so called because they supply nutrients to the developing egg, and are relatively large cells measuring 18-22µm, with an irregularly shaped nuclear membrane (Plate 2.5). The number of the nurse cells is species specific (Buning, 1994) and in *L. bostrychophila* three of the daughter cells become the nurse cells while the other differentiates into an oocyte. In the region of the nurse cells, the prefollicular epithelium (follicular cells) is very thin. In early stages of oocyte growth, the follicles grow inward between the nurse cells and the maturing oocyte, but do not separate them completely (Plate 2.4).

2.4.6 Follicle Cells

At later stages the oocyte of *L. bostrychophila* becomes completely surrounded by a single cell layer of follicle cells (Plate 2.6). These surround the growing oocyte during the early cytoplasmic growth phase and shortly before vitellogenesis occurs. At this stage the nuclei become amoeboid and the cells increase greatly in size as they become

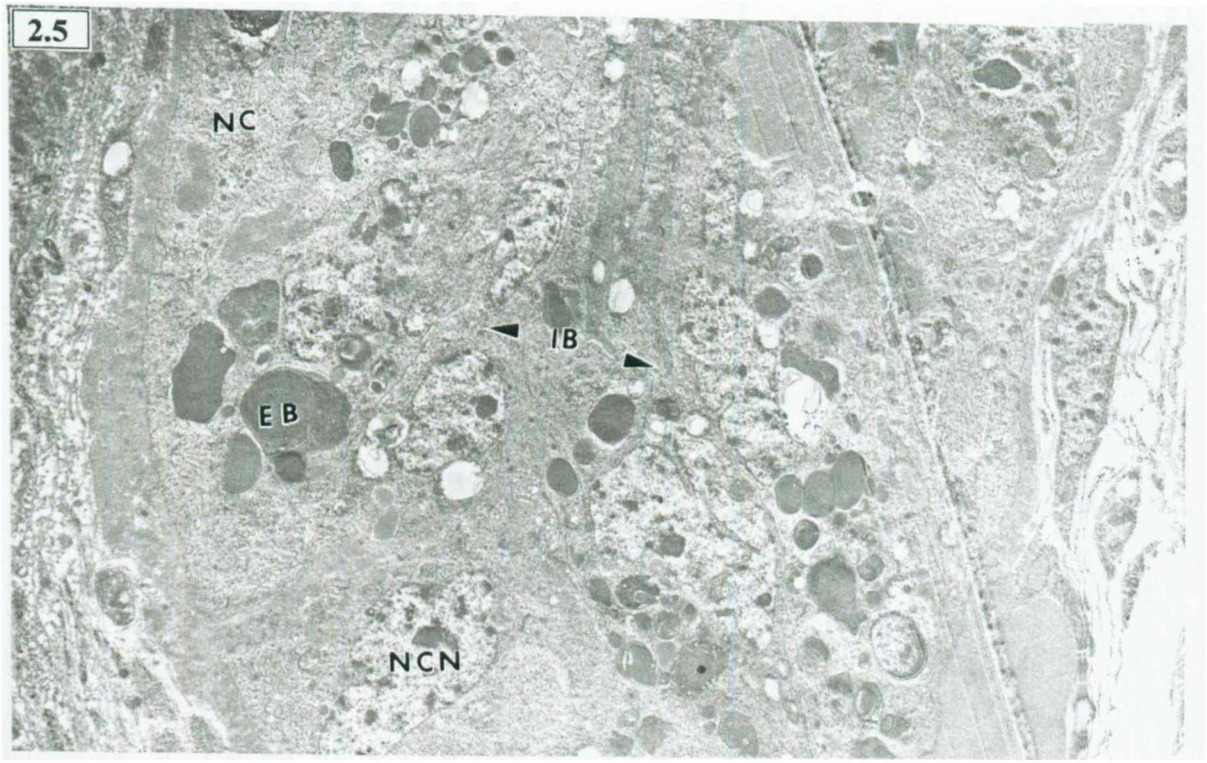


Plate 2.5. Nurse cells ultrastructure of *L. bostrychophila*. The large nuclei of the nurse cells are heavily enfolded and the nuclear material is shown in this micrograph as isolated pockets. The nurse cell cytoplasm is of low electron density similar to that of the early cytoplasm of the oocyte. NC, Nurse Cells; NCN, Nurse Cells Nuclei; IB, intercellular Bridges; EB, Endobodies; Magnification 5800x.

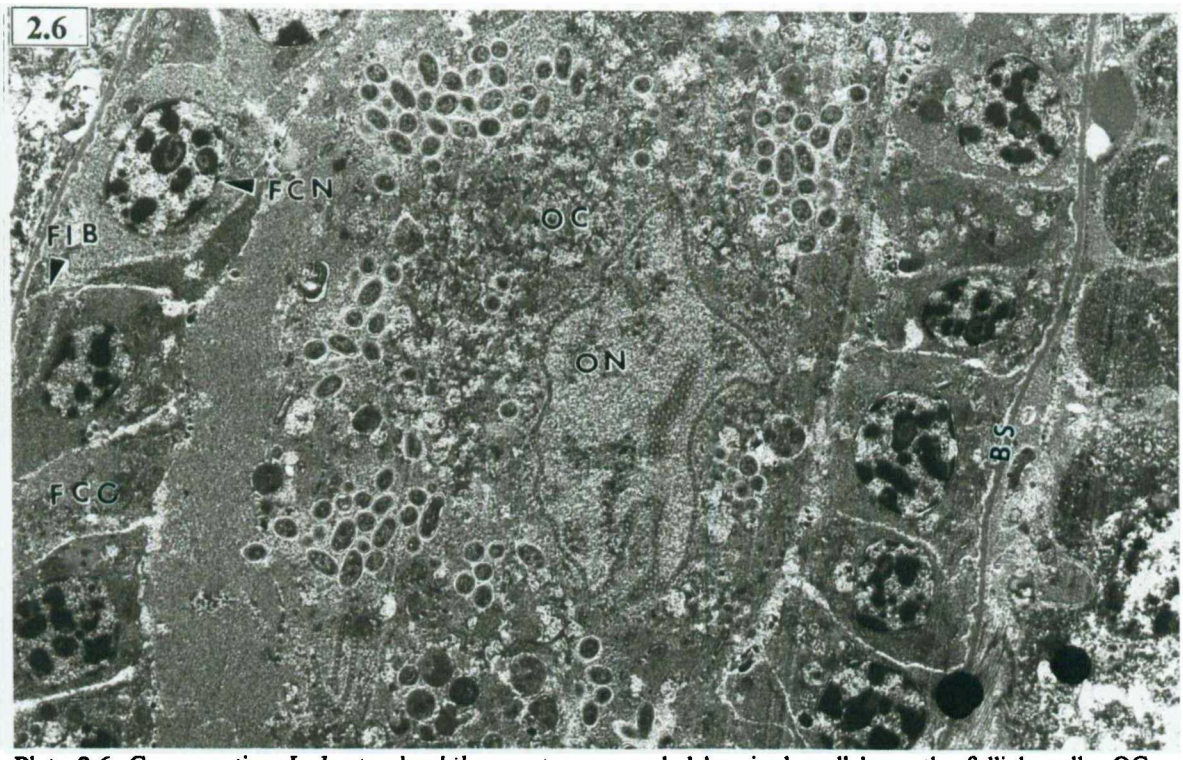


Plate 2.6. Cross section *L. bostrychophila* oocyte surrounded by single cell layer the follicle cells. OC, Oocyte; BS, Basement Membrane; ON, Oocyte nucleus; OC, Oocyte Cytoplasm; FCN, Follicle Cell Nucleus; FCC, Follicle Cells Cytoplasm; FIB, Follicle Intercellular Bridges; Magnification 5800x.

active partly because of vitellogenesis, but primarily in the secretion of the protective covering of the egg (chorionogenesis).

Follicle cells are assembled in three morphogenetic zones with functionally integrated components. This are the follicle cytoplasm, the follicle cell epithelium (basement membrane) surrounding the oocyte and the intercellular spaces of follicle cells, the ring canals (Plate 2.6, 2.7). These meshwork is thought to serve as a channel for the influx of precursors as well as the meeting ground for nutrients from blood and those secreted by the follicle (Kunkel, 1986). These will in turn differentiate into the different layers of the eggshell. Terms like basement membrane (tunica externa/propria) and follicle-oocyte border referring the outer and inner follicular cell membrane respectively are used interchangeably in describing liposcelid follicular ultrastructure.

The structure and thickness of follicle cells varies from species to species. In *L. bostrychophila* the internal portions of the follicular cell membrane) and the developing oocyte cell membrane are highly porous (Plate 2.7).

These pores have ring shaped structure that are up to 86ηm apart suggesting an extensive transfer of nutrients to the oocyte. Woodruff and Telter (1990) demonstrated that within each follicle an electrical gradient exists in all types of insect ovarioles, by which cytoplasmic components may be ordered asymmetrically in the egg, according their own charge. The internal membrane of the follicle cells posses numerous intercellular bridges between follicle cells and the oocyte. Material produced by the follicle cells is visible inside the follicles as well as in the oocyte cytoplasm (Plate 2.7).

The nuclei of the follicle cells are very conspicuous and their chromatin material is rather diffuse indicating actively secreting epithelial cells. High magnification of different EM sections in the *L. bostrychophila* oocyte shows the porosity of the inner follicular membrane. The oocyte, when in the vitellogenic stage, shows increased deposition of yolk and lipid material in the egg cytoplasm (Plate 2.7).

2.4.7 The oocyte

The most dominant feature in an oocyte of *L. bostrychophila* is the oocyte nucleus with its irregular, porous and amoeboid shaped nuclear membrane, through which it communicates with the surrounding cytoplasm (Plate 2.8). The oocyte cytoplasm is filled

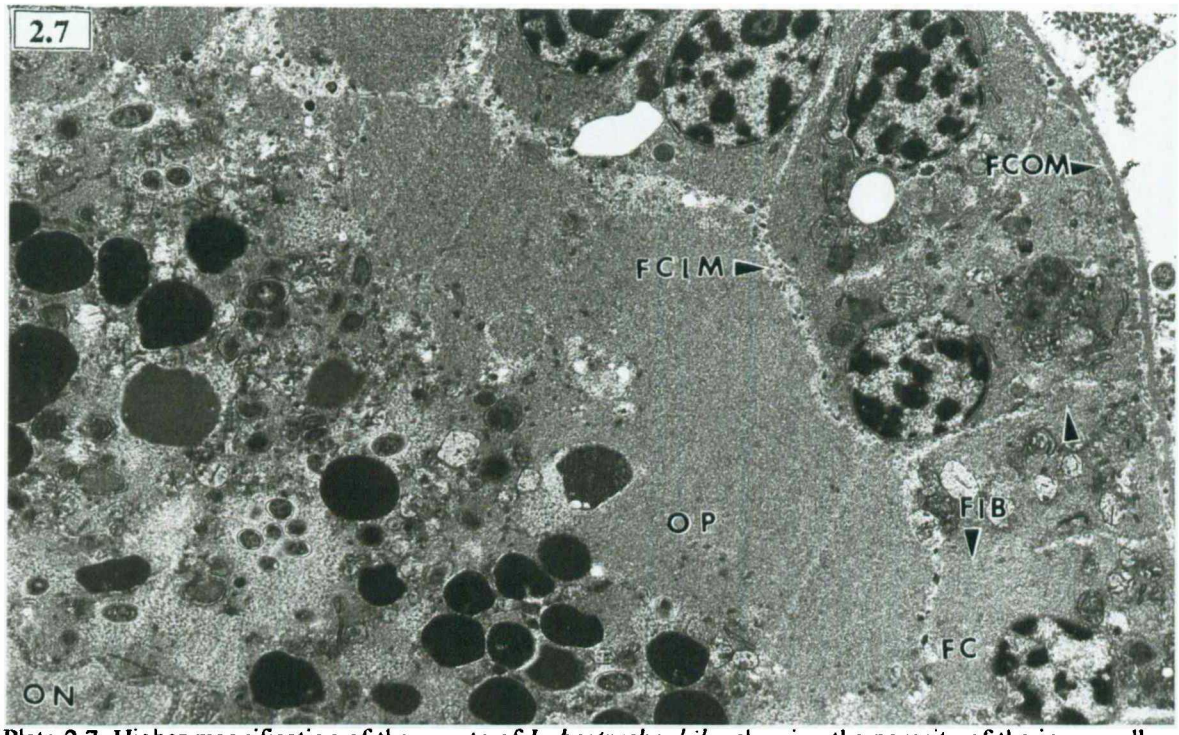


Plate 2.7. Higher magnification of the oocyte of *L. bostrychophila*, showing the porosity of the inner wall of the follicular cell bordering the oocyte. Follicle cells are interconnected with each other through the follicle intercellular bridges (FIB). FCOM, Follicular cell outer membrane; FCIM, Follicular cell inner membrane; ON, Oocyte nucleus; OP, Oocyte Cytoplasm; FC, Follicle Cells. Magnification 5800x.

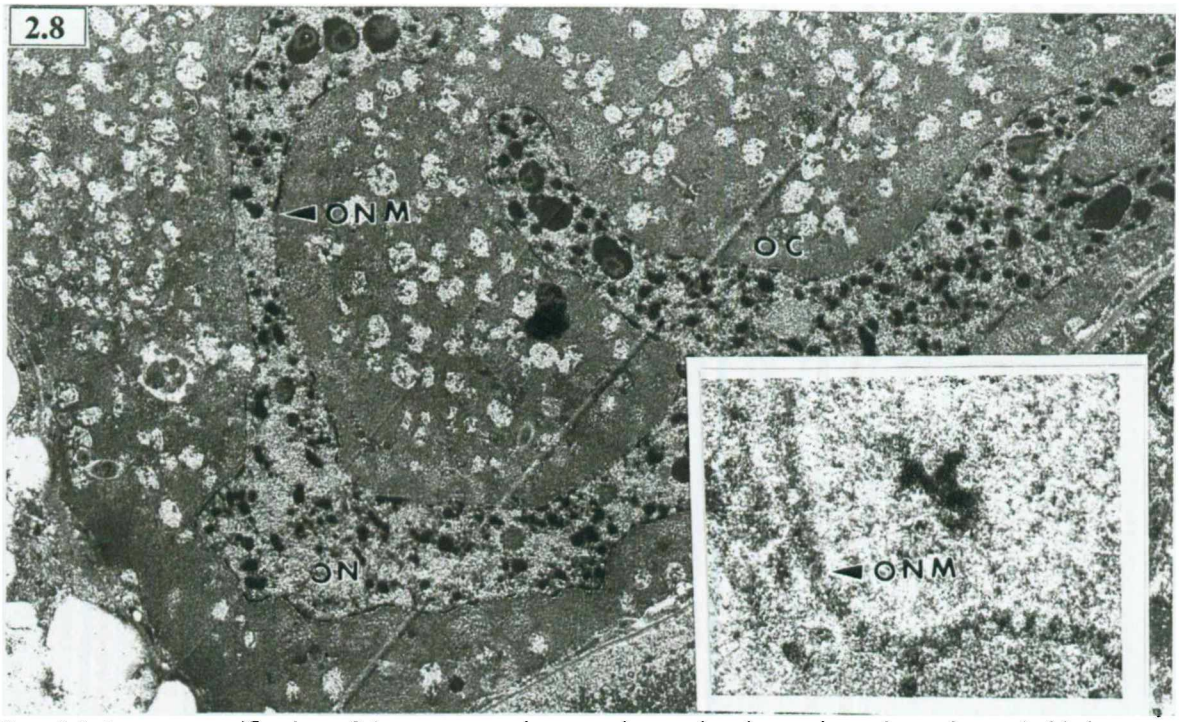


Plate 2.8. Lower magnification of the oocyte nuclear membrane showing an irregular and amoeboid shape through which it communicates with the surrounding cytoplasm. The insert is high magnification (28000x) of the oocyte nuclear membrane of *L. bostrychophila*. ONM, Oocyte Nuclear Membrane; ON, Oocyte nucleus; OC, Oocyte Cytoplasm; Magnification 5800x.

with vacuoles, endoplasmic reticulum, ribosomes, mitochondria, yolk material and other inclusions together with the large nucleus.

As the egg matures oogenesis is completed, the deposition of yolk ceases and chorionogenesis starts, whereby the same nurse cells produce chorionic material for the construction of the eggshell. Subsequently the chorion hardens, and mature egg is laid.

2.4.8. Ovariole ultrastructure, oocyte differentiation and oogenesis

In *L. bostrychophila* ovarioles go through various developmental stages to produce a mature egg. These include oocyte-nurse cells differentiation, previtellogenesis, vitellogenesis and finally the chorionogenesis (vitelline membrane and chorion deposition) as the oocyte develops. Egg growth is split into two tightly coupled events, namely the cytoplasmic (previtellogenesis) and the vitellogenic growth.

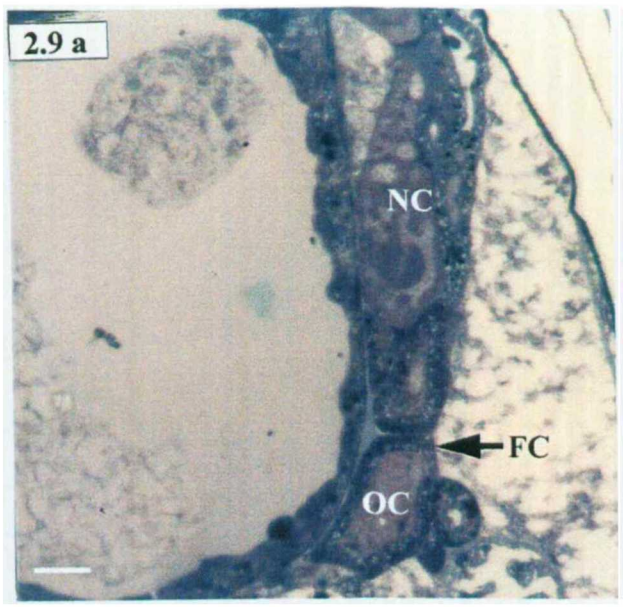
2.4.9. Previtellogenic stage of the oocyte

The most anterior part of the ovariole in this species, the cystocytes, is indistinguishable. Only profuse and dividing cells can be observed in this part of the germarium. These cells are the somatic tissues of the germarial region. The somatic cells at the tip of the germarium lying posterior to the transverse septum, divide the terminal filament from the germarium and form a monolayered sheath. This area enlarges to accommodate the previtellogenic growth of the germ cell descendants namely, the nurse cells and the oocyte. The nurse cells grow simultaneously with the oocyte with which they transfer cytoplasmic macromolecules. In this species the differentiation of oocytes and the nurse cells occur relatively late. The nurse cells are interconnected with themselves and with the developing oocyte through the ring canals (Plate 2.10).

The previtellogenic growth of psocid ovarioles can be divided in to five developmental stages (Buning and Sohst, 1988):

Stage I. Cystocytes do not grow but divide mitotically and the four cell clusters maintain minimum volumes (Plate 2.9a).

Page 61 - Plate 2.9 overleaf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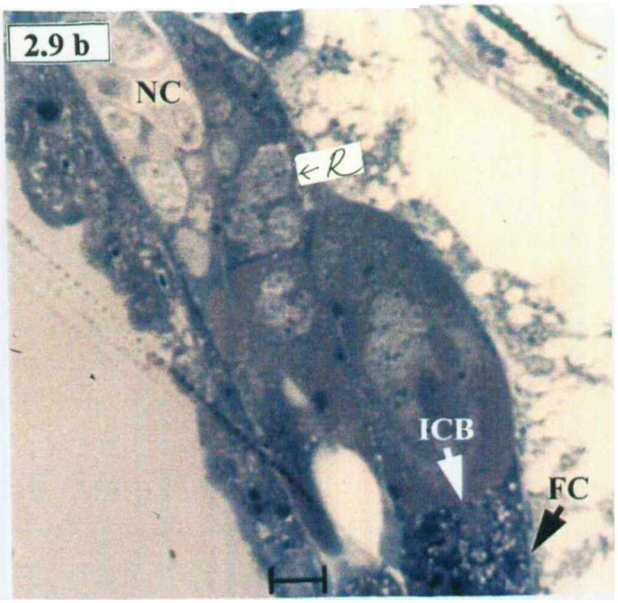
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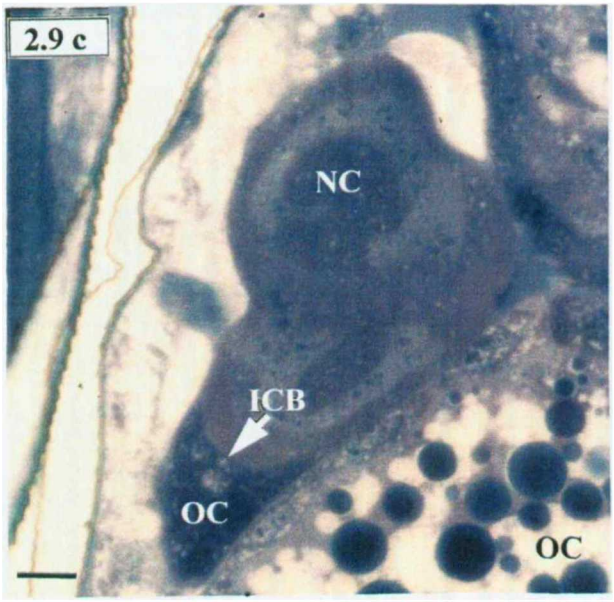
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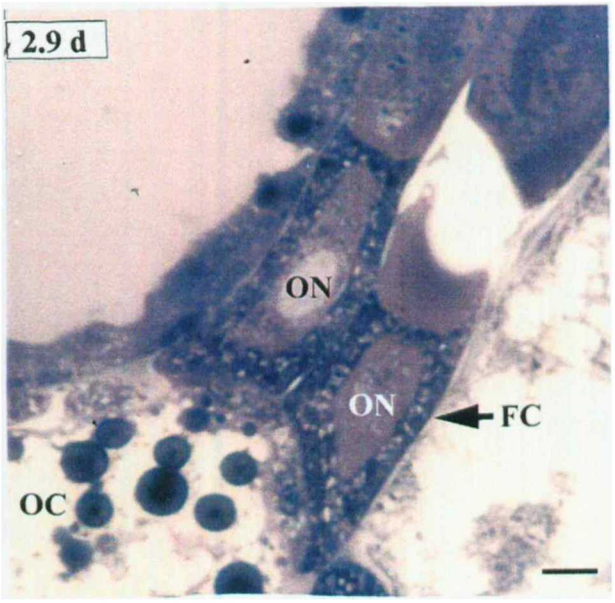
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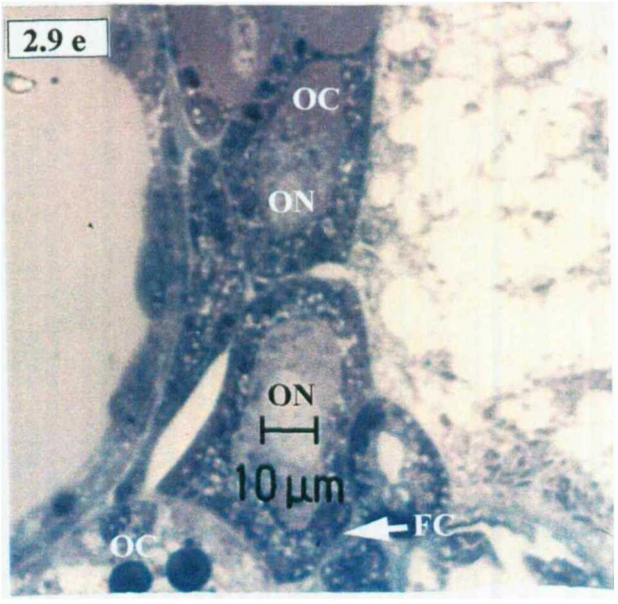
2.9 e
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Stage II. Growth proceeds when all four cystocytes enter meiotic prophase and the formation of the rosette. At this stage the ooplasm of the previtellogenic oocyte is similar to that to the nurse cell cytoplasm. Hence it is impossible to distinguish the prospective oocyte (Plate 2.9b).

Stage III. This stage occurs in the lower part of the germarium. All four cells lose their synaptonemal complexes. During this phase the three nurse cells grow rapidly, while the oocyte grows very slowly (Plate 2.9c).

Stage IV. As the oocyte continues to enlarge the nurse cells growth rate slows (Plate 2.9d).

Stage V. This is the shortest of all previtellogenic stages. At the end of the previtellogenic growth, nurse cells become integrated into the oocyte and eventually break down. The oocyte receives cytoplasm from the nurse cells and oocyte size increases further (Plate 2.9e).

The main changes of previtellogenic stage in the ovarioles of the booklouse occur in the oocyte nucleus. In the previtellogenic stage the size of the oocyte cytoplasm is markedly smaller, measuring 5μm in early previtellogenesis and growing to 25μm in length at late previtellogenesis. The oocyte also shows the intercellular bridges (ring canals) between nurse cells and growing oocyte (Plate 2.9c).

The previtellogenic growing oocyte of *L. bostrychophila* is accompanied by many follicle cells arranged in highly columnar fashion. In the middle of the previtellogenic stages, the follicle cells become elongated in a roughly cuboid shape. It is this stage when follicle cells grow intensely. In the polytrophic ovaries of *L. bostrychophila* after the cystocytes have differentiated and the oocyte has become surrounded by follicle cells, all the chromatin becomes clumped in one region, the karyosome, which is about 0.5μm in diameter. The previtellogenic follicle cells have relatively low DNA/chromatin content (Plate 2.11).

The nurse cells are first differentiated from the oocyte when irregularly dispersed chromatin granules can be seen in their nuclei and nuclear membrane begin to fold, Whilst the oocyte assumes an ovoid shape with thread-like chromatin material. During previtellogenesis the nurse cells, which were physically attached to the anterior tip of the developing oocyte, become integrated.

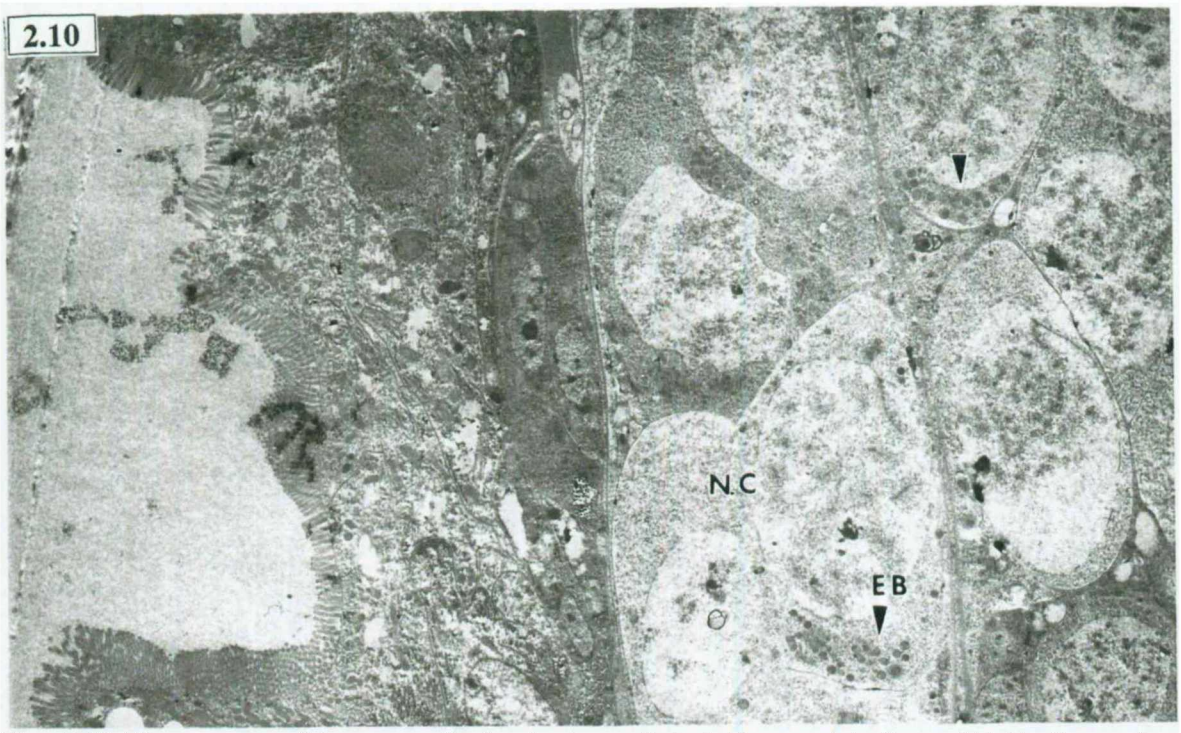


Plate 2.10. Ultrastructure of the nurse cells showing intercellular bridges, endobodies and highly dispersed state of their chromatin material. NC, Nurse cells; EB, Endobodies. Magnification 3800x.

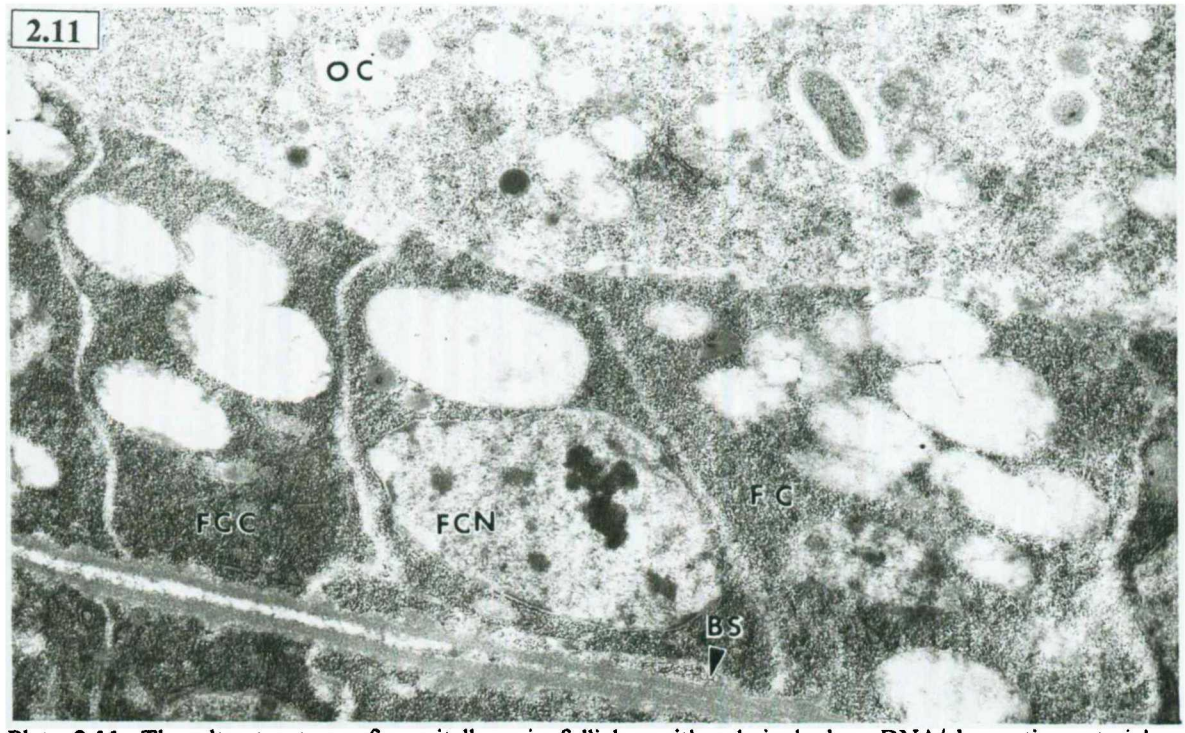


Plate 2.11. The ultrastructure of previtellogenic follicles with relatively low DNA/chromatin material content. FC, Follicle Cells; FCN, Follicle Cell Nucleus; FCC, Follicle Cells Cytoplasm; BS, Basement Membrane; OC, Oocyte Cytoplasm. Magnification 11500x

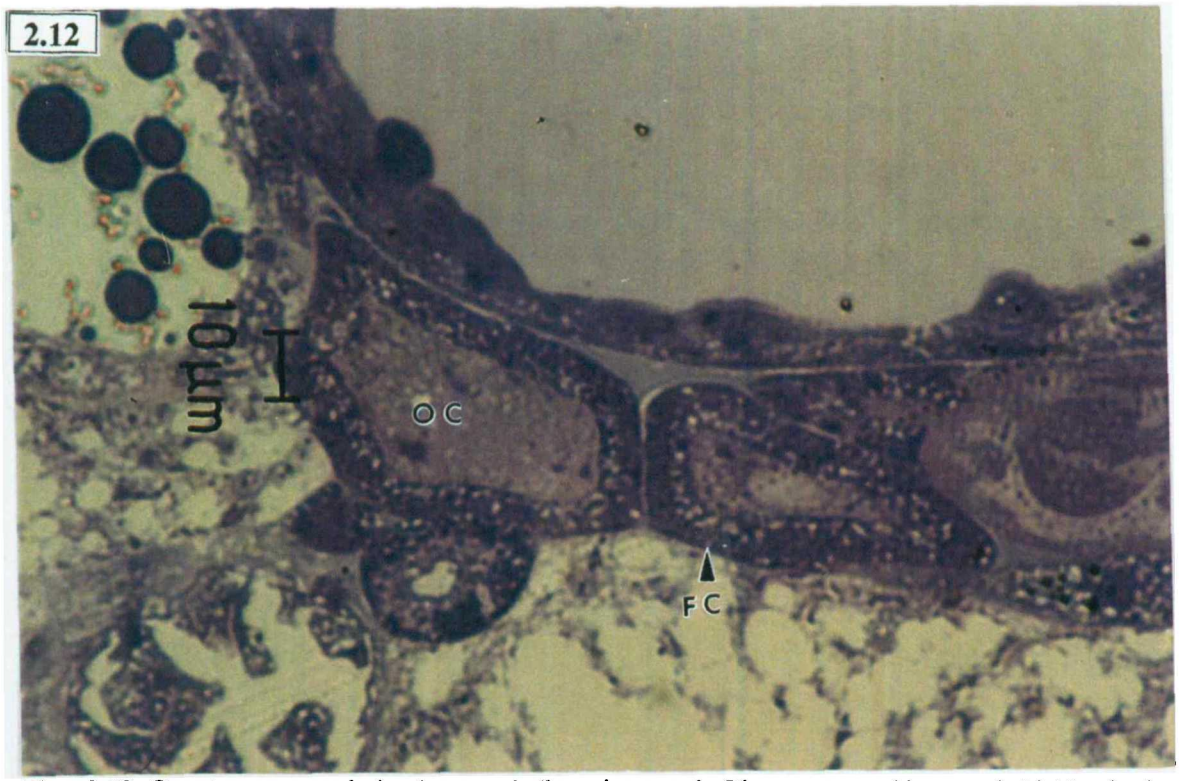


Plate 2.12. Oocyte structure during late previtellogenic growth. The oocyte at this stage is highly elastic and shape is dictated by the spatial arrangement of other components in the female abdomen. OC, Oocyte; FC, Follicle Cells.

Plate 2.13. The oocyte of *L. bostrychophila* at the beginning of vitellogenesis as the first droplets of vitellogenins appear as dark globules in oocyte cytoplasm. OC, Oocyte; FC, Follicle Cells.

At this stage the oocyte gradually changes shape from conical to ovoid surrounded by uninucleate follicle cells with three stretched, barely distinguishable nurse cells dorsally (Plate 2.12). The oocyte is in this stage still elastic with variable shapes, dictated by the other abdominal inclusions.

The zone of previtellogenic growing oocytes ends with the oocyte just beginning vitellogenesis.

2.4.10. The vitellogenic stages

The vitellogenic growth phase begins when yolk spheres are first observed to accumulate in the oocyte (Plate 2.13). When vitellogenesis starts in the oocyte the nurse cells breaks down immediately and the young oocyte subsequently enlarges by the incorporation of nurse cell material and follicle cell secretions. EM micrographs of the ovarioles of *L. bostrychophila* show the follicular cells forming a monolayer surrounding each growing oocyte. The follicle cells are the site of yolk synthesis and can be seen by the presence of droplets (0.2-0.5μ) of yolk forming inside them. These droplets move towards egg cell membrane (Plate 2.14) and aggregate into relatively larger globules of various sizes, measuring up to 5μm, inside the developing oocyte (Plate 2.14).

At the beginning of vitellogenesis the follicular cells are relatively small, measuring 3.5μm across (Plate 2.15). The follicular cells grow in size during late previtellogenesis and early vitellogenesis when there is an enlargement of the nucleic material and other cytoplasmic organelles (Plate 2.16). The DNA content of the follicle increases during this growth phase of the oocyte.

Each oocyte is surrounded by a fixed number of follicle cells. In cross section the oocyte circumference has 30 follicle cells attached to it. The number of cells appears to stay the same throughout the oocyte development although occasional division was observed. However their size more than triples as the egg grows.

In *L. bostrychophila* it appears that follicle cells are interconnected with intercellular bridges (Plate 2.14 and 2.16). These bridges make communication between these cells possible, and are assumed to play a key role in the co-ordination of functions of the follicle cells during the synthesis and secretion the eggshell components (chorion and vitelline membrane) and in the accumulation of yolk materials by the oocyte.

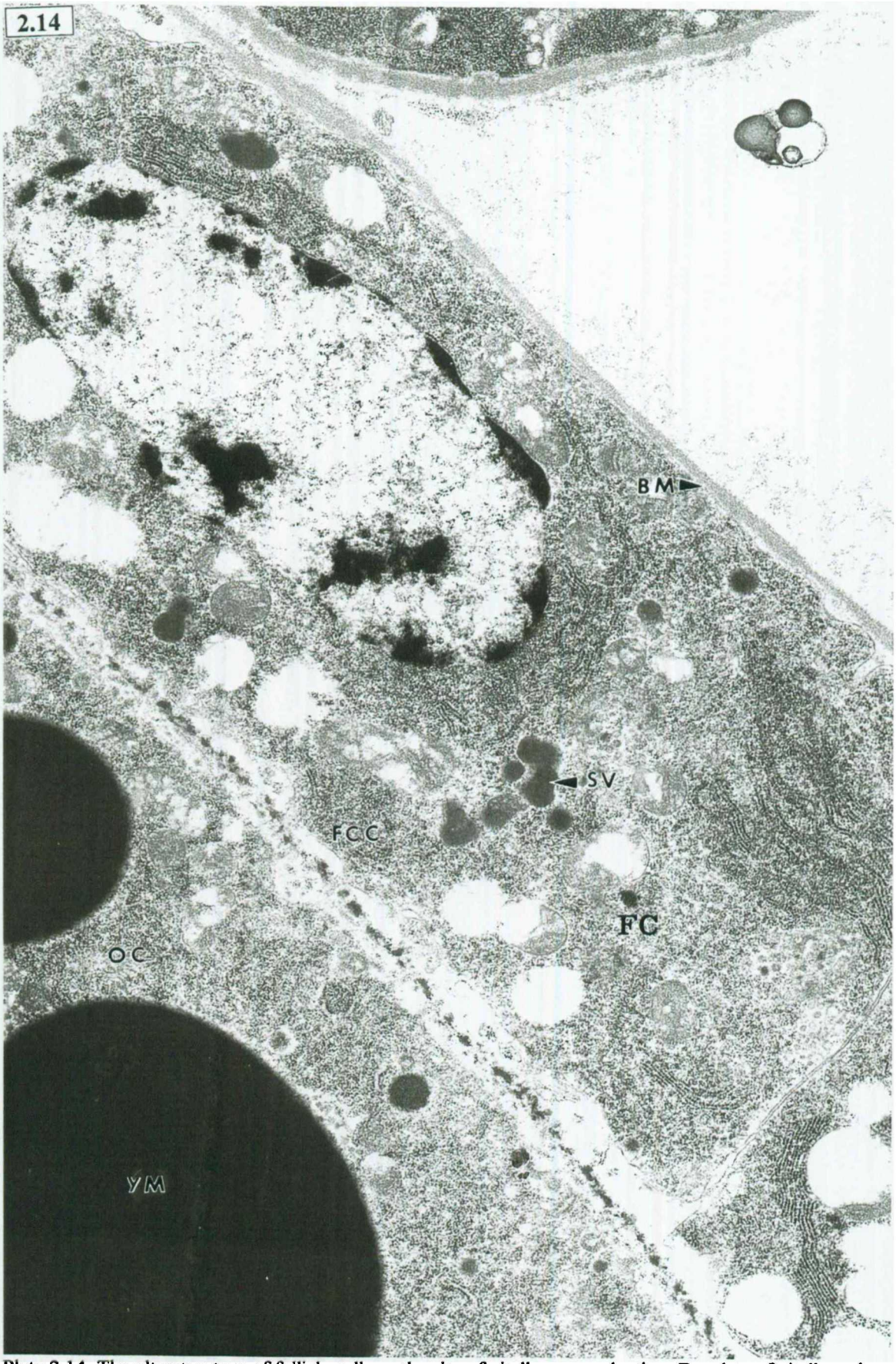


Plate 2.14. The ultrastructure of follicle cells as the site of vitellogen production. Droplets of vitellogenins of similar size and electron density appear inside the follicular cytoplasm and into oocyte cytoplasm. FC, Follicle Cells; FCC, Follicle Cell Cytoplasm; YM, yolk Material; SV, Secretory vesicles; OC, oocyte Cytoplasm; BM, Basement Membrane. Magnification 21600x.

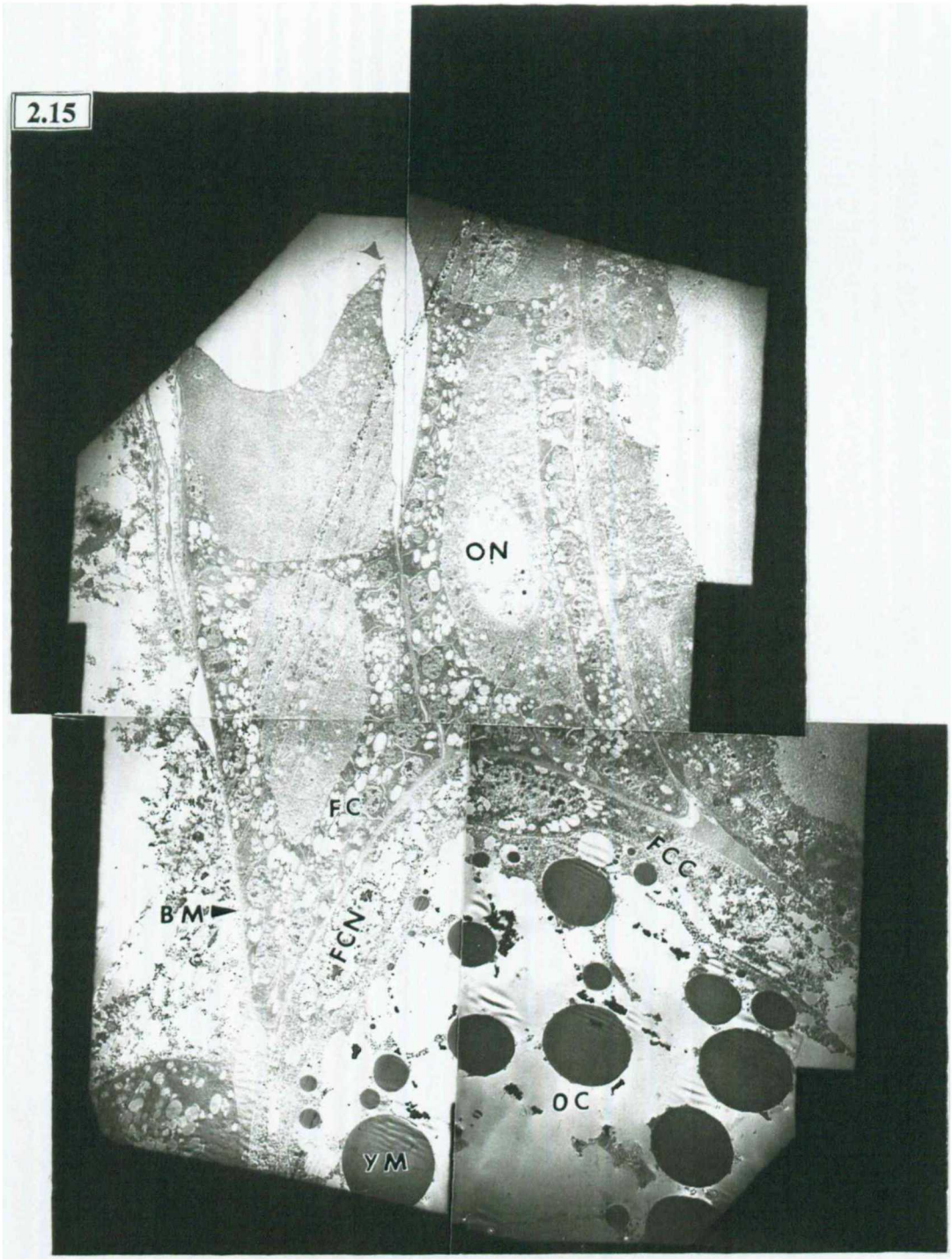


Plate 2.15. This plate shows oocytes in several stages of development from previtellogenesis through to completed vitellogenesis. Part of a fully developed and expanded oocyte occupies the lower part of the plate, with two small previtellogenic oocytes above.
BM, Basement Membrane; ON, Oocyte nucleus; OC, Oocyte Cytoplasm; FC, Follicle Cells; FCN, Follicle Cell Nucleus; FCC, Follicle Cells Cytoplasm; YM, Yolk Material. Magnification 1400x.

Plate 2.16. High magnification electron micrograph of a vitellogenic oocyte in active secretion. At this stage the follicles show pronounced nuclear material and nuclear division (Cell wall shared) is seen in the upper cell. FCC, Follicle Cell Cytoplasm; FCN, Follicle Cell Nuclei; OC, Oocyte Cytoplasm.; SV, Secretory Vesicle. Magnification 21600x.

The size of the yolk globules varies with the oocyte age and between individuals.

The lipid yolk spheres occur at the onset of the vitellogenesis (Plate 2.13 and 2.14).

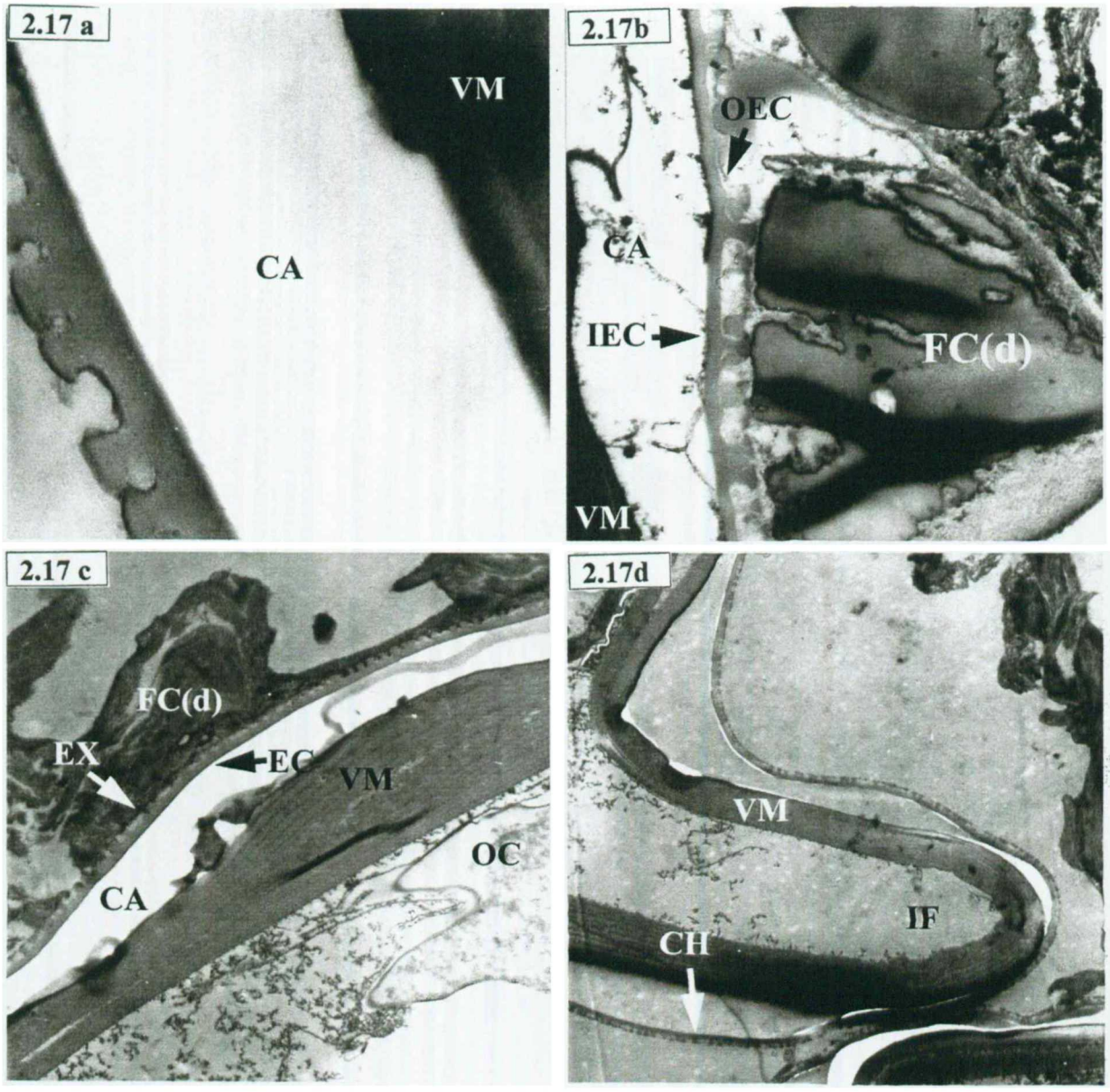
These macromolecules (lipid and proteaceous yolk) are stored in vacuoles. The oocyte cytoplasm increases until the oocyte extends across the whole width of the ovariole and has been surrounded by a follicular epithelium.

In the later stages of vitellogenesis growth is mainly restricted to cytoplasmic components. Initially small yolk globules measuring 0.5-1.5μm appear along the follicular border but they soon fill the ooplasm, measuring up to 11.5μm in some late vitellogenic oocytes. The ultrastructural data on *L. bostrychophila* have shown the pronounced nuclei in follicular cells measuring 2.5-3.5μm during late previtellogenic and vitellogenic stages, an indication of their high rate of synthesis (Plate 2.16). The DNA content of the follicle cells is raised during the growth phase of the oocyte. Follicles assume a high nuclei-cytoplasm ratio and occasionally dividing nuclei are seen. Follicle cells, during yolk deposition and vitelline membrane production, typically have intercellular spaces between them. Their nuclei are centrally located with scattered clumps of chromatin. Follicle cells in this stage show an increased number of ring-shaped secretory vesicles (Plate 2.16) and contribute to the formation of the vitelline membrane (VM).

The dimensions of the karyosome (oocyte nucleus chromosomes) gradually increase during subsequent growth stages of the oocyte until the stages of the vitellogenesis when the karyosome is completely spread out.

Follicle cells change their form and function several times, from producing macromolecules for the developing oocyte,to producing a new set of molecules which will form the vitelline membrane and the chorion. The completion of vitellogenesis in *L. bostrychophila* eggs is not only marked with the increase of the oocyte in size and the termination of deposition yolk globules, but also the production of the vitelline membrane (Plate 2.17a). The vitelline membrane is the first layer surrounding the oocyte plasma membrane and is also the first product of the follicle cells in terms of eggshell synthesis, since these cells are also engaged in the synthesis of yolk globules.

Page 71 - Plate 2.17 overleaf


2.17 a
VM
CA
2.17b
OEC
CA
IEC
FC(d)
VM
2.17 c
FC(d)
EX
EC
VM
OC
CA
2.17d
VM
IF
CH

Initially the vitelline bodies form strings of electron dense bodies that accumulate at the interface between the follicle cells and the oocyte (Plate 2.17b). These bodies fuse subsequently and in later stages transform into a continuous membrane layer measuring 1.0μm surrounding the whole egg (Plate 2.17c).

As the oocyte volume has increased so the vitelline membrane has become thinner as it stretches (from 3.0 to 0.5μm). The vitelline membrane has finger-like infoldings, which extend to a depth of 30μm within the ooplasm (Plate 2.17d).

2.4.11. The mature ovary eggs

The main features of the maturing oocyte have been described in relation to Plate 2.6. The oocyte cytoplasm contains a variety of inclusions including electron dense spherical bodies up to 1.7μm in diameter and larger inclusions, which are 2.0-2.5μm in diameter with homogeneous, less electron dense contents. Plate 2.6 and 2.8, shows the cytoplasm matrix organised in a mosaic fashion with much of the matrix showing a fine granular appearance of moderate electron density interspersed with 'islands'' of reduced electron density containing coarser granular polyribosome aggregates.

In later phases of vitellogenesis the oocyte membrane becomes fused with the vitelline layer by which time transmembrane communications will cease.

The oocyte of *L. bostrychophila* does not have invaginations of the oocyte border forming structures that have been called micropinocytotic invaginations or pitted vesicles. These vesicles have been observed in many insect species (Telfer and Smith, 1970). In *L. bostrychophila* however, structural relationships between the follicle cells and the oocyte change. The internal oocyte border is always convex-shaped and lacks any invagination between the oocyte and surrounding follicle cells. This relatively simple architecture contrasts with oocytes of insects from higher orders.

For example in the *Aedes aegepti* a blood meal triggers the start of the vitellogenesis and the surface of the egg is surrounded with large number of these invaginations. The numerous gaps and septate junctions present between follicle cells and oocyte largely disappear at the end of vitellogenesis and the beginning of the formation of the vitelline membrane (Buning, 1994).

As the liposcelid egg reaches maturity the karyosome (DNA-chromatin material of the oocyte nucleus) gets very dispersed. Yolky components comprise most of the volume of the mature egg. Nevertheless eggs at the same stage of development often contain markedly different amount of yolk globules. This is due to different loads of bacterial endosymbionts, which is described, in the following chapter.

2.4.12. The chorion

Throughout the next developmental stages, up to when the egg is laid, the oocyte volume does not change significantly and the hard and non-expandable outer layer the eggshell (chorion) is deposited (Plate 2.17b).

Scanning electron micrographs of laid *L. bostrychophila* eggs reveal a complex surface sculpturing (Plate 2.18a). The sculpturing is "plastron" like made of rounded and mushroom shaped structures in which the 'caps' partially fuse together to create a patchwork surface with spaces connecting with the sub-exochorion air spaces.

In section the chorion is multilayered, having an innermost chorionic layer, the endochorion and the outer exochorion. The innermost chorionic layer is very thin measuring 0.2μm. A cavity 1.5μm wide separates this layer from the vitelline membrane at the bottom. The endochorion is the thickest region of the chorion and it measures 2-2.8μm. It is a continuous and uniform layer lacking any regional complexity or ridges unlike the endochorion of other comparable insect species. The exochorion measures 1.5-2μm, and is disrupted by holes varying between 0.5-3.5μ in diameter. It shows as a comb-like structure in sections (Plate 2.17a). These holes may serve as aeropyles, which communicate with the sub-exochorionic space beneath it.

The eggshell of *L. bostrychophila* lacks any specialised respiratory appendages of the type present in some insect eggs. The structure and the thickness of the insect eggshell vary; in *L. bostrychophila* it is thin measuring 4μm.

Thin section electron microscopy of the ovary at this stage shows the follicle cells becoming rounded and degenerating (Plate 2.17b and 2.17c). The chorionogenic follicle cells possess large cavities; the edges of the cavities coincide with the transverse follicular membrane separating between two adjacent cells, which is also degenerating. When the first egg is ready to be laid, the remaining follicle cells collapse to globular

bodies together with the encompassing tunica propria. An irregularly shaped, electron dense area is revealed on the outer surface of the exochorion layer. Vacuoles form between the chorion and the egg content (Plate 2.17b and 2.17c). Although reproduction is by parthenogenesis the egg still possesses a well-developed micropylar structure, the site of sperm entry in bisexual insects.

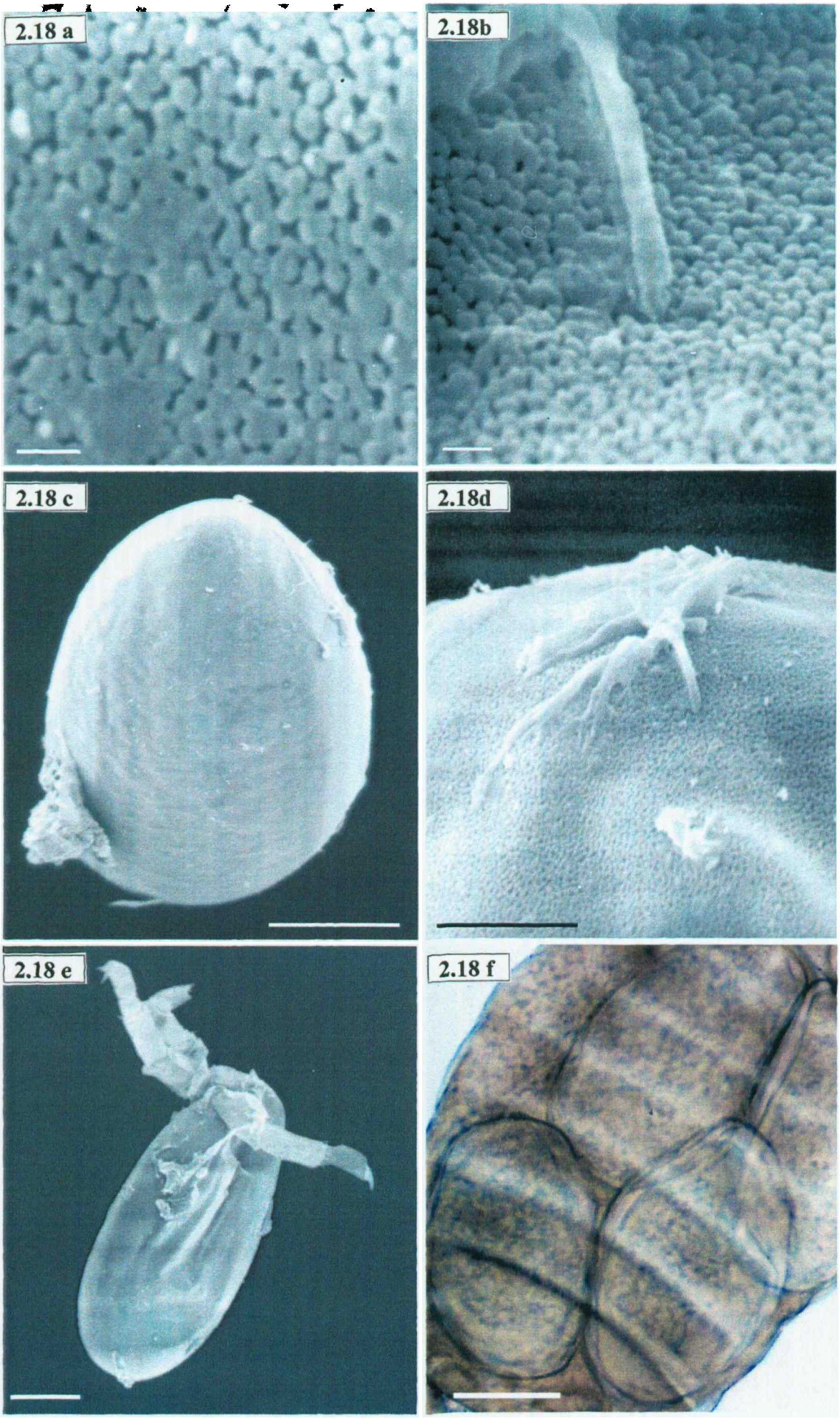
The neatly arranged and compact nature of the chorion might afford the embryo some degree of protection from the deleterious conditions such as unfavourable humidity and temperature while allowing gas exchange between inside and outside of the egg. It also prevents fixatives penetrating the egg. The micropyle/areolate is positioned towards the more tapered end of the egg (Plate 2.18b). This is a weak pressure point where the egg cracks along the micropyle line to let the nymph emerge.

Dissected mature ovary eggs of *L. bostrychophila* are soft and easily deformed. Once the deposition of the eggshell (chorion) ends the sclerotisation of the egg starts, suggesting that the process of sclerotisation and melanization or so called 'hardening' and 'darkening' are completed after the egg is being laid. Females become stationary just prior to oviposition. The tip of the abdomen assumes a curved position, and a repeated tapping movement is then observed. A single egg is extruded and deposited in crevice or on rough surfaces.

2.4.13. The laid egg of *L. bostrychophila*

Eggs are ovoid in shape and slightly pointed at one end (Plate 2.18c). In this psocid species the eggs are translucent, iridescent, laid singly and firmly attached to the substrate (filter paper in laboratory culture conditions) by means of a sticky material which coats the whole egg. Particles of dust and food adhere to the sticky egg surface and camouflage it. New (1974) proposed that this sticky material together, with sculpturing, which is widespread among Psocoptera, provides the egg with addition protection from desiccation. The freshly laid eggs of *L. bostrychophila* are also bilaterally symmetrical and the surface sculpturing and ridges are concentrated around the tapered end of the egg, the anterior pole (Plate 2.18d). The anterior pole shows a pronounced micropyle with radiating filamentous projections measuring 10-20μm in length.

Page 76 - Plate 2.18 overleaf


2.18 a
2.18b
2.18 c
2.18d
2.18 e
2.18 f

The chorion of the newly laid egg is soft. This hardens in the air and later begins to show signs of collapse, probably due to some water loss (Plate 2.18e).

The total volume of the egg shrinks increasing the pressure on the anterior pole of the egg where the nymph emerges, in legs first position (Plate 2.18e).

2.4.14. Egg size

Body size and to a lesser extent egg size, in psocids is variable. More importantly psocids differ in the size of their eggs relative to their body size and weight. Liposcelids are among the smallest psocids but their eggs are not greatly different in size from those of other species. This results in *L. bostrychophila* having the largest recorded ratio between egg and adult size. *Liposcelis bostrychophila* eggs measure an average of length 0.34mm *(±2SE = 0.02)* by width 0.19mm *(±2SE = 0.03)*, and represent approximately 1/3 of the length of the adult. In comparison the eggs of *Trichadenotecnum (=Psocus) sexpunctatus* the egg is less than 1/14 of the body size.

Larger psocid species do not lay commensurately larger eggs and so have far smaller egg to adult size ratios, however some larger species (*Stenopsocus stigmaticus, S. immaculatus, Ectopsocus briggsi, Graphopsocus cruciatus* and *Caecilius* sp.) lay eggs in small batches of up to 16 eggs (New, 1969). Pearman (1928) compared the relative sizes of eggs and adults in some psocids and presented ratios of length of the egg to that of the female. These ratios appear to be in error and have recalculated from Pearman's size/length data (Table 2.1).

Plate 2.18f, shows the distended abdomen of gravid female of *L. bostrychophila.* Eggs occupy most of the abdomen. The number of eggs shown in this figure represents the maximum seen. Normally 1-2 eggs are visible from the abdomen in freshly mounted specimens.

The majority of psocids are winged. Body length is quite variable depending on the fullness of the abdomen with food or eggs, hence the length of the forewing might be considered a more accurate measurement of the adult female size. Ratios between psocid eggs to the length of the forewings were calculated and shown in Table 2.2.

Hinton (1981), also suggested that a more meaningful comparison would be the ratio of the dry weight of the females to that of their eggs.

L. bostrychophila can produce eggs weighing its own dry mass per day. Egg and adult weight measurements are available only in *L. bostrychophila* (Table 2.1). The water content of *L. bostrychophila* eggs is also high, measuring 83.6-84.4%, the rest represent egg dry material including the chorion (Table 2.1).

The high water content of *L. bostrychophila* eggs coupled with high egg output, creates a particular challenge to the water balance in this insect living in a dry environment (dry and stored products). A large proportion of water in *L. bostrychophila*'s body is devoted to the egg production.

Psocid species	Mean egg length (mm)	Adult length (mm)	Ratio egg: adult	Mean egg Width (mm)	Wet weight/ 20 eggs (mg)	Dry weight/ 20 eggs (mg)	Wet weight/ egg (mg)	Dry weight/ egg (mg)	Water content (%)	Max.no. eggs/ female/day
Liposcelis bostrychophila	0.35	1.00	1:3	0.185	0.718	0.16	0.0359	0.008	77-71	3-4
Lachesilla pedicularia	0.37	3.33	1:9							
Reuterela helvimaculata	0.43	3.67	1:8.5							
Elipsocus westwoodi	0.47	5.00	1:11							
Psocus sexpunctatus	0.41	6.00	1:15							
*Psocus longicornis**	0.57	10.0	1:18							

Table 2.1. Weight, egg size, egg to adult ratio, comparative female size and water contents of the eggs of *Liposcelis bostrychophila* and in some other psocid species. The data on psocid species other than *L. bostrychophila* are recalculated figures (Pearman 1928).
*Renamed *Psococerastis gibbosa.*

Psocid species	Mean fore wing length (mm)	Mean egg length (mm)	Ratio Length Fore wing:egg	Eggs laid in
Cyanops cyanops	2.55	0.490	1:5	Batches
Philatarsus picicornis	3.40	0.488	1:7	"
Elipsocus hyalinus	3.00	0.468	1:6.5	"
E. Mclachlani	2.40	0.456	1:5.5	"
E. westwoodi	3.00	0.485	1:6.5	"
Trichopsocus dalii	2.70	0.476	1:6	Single
Ectopsocus briggsi	--	0.246	--	Batches
Stenopsocus immaculatus	5.25	0.630	1:8	"
S. stigmatus	4.25	0.580	1:7	"
Graphopsocus cruciatus	3.20	0.464	1:7	"
Caecilius flavidus	3.00	0.438	1:7	"
C. burmeistri	2.65	0.426	1:6	"
C. fuscopterus	3.50	0.430	1:8	"
C. kolbi	2.80	0.415	1:7	"

Table 2.2. Comparative ratios of mean egg length and fore wing length measurements in some winged psocids. Data extracted from Pearman (1928), Broadhead (1950), New (1969)

2.5. DISCUSSION

The central focus of this chapter is to describe the gross morphology and the ultrastructure of the reproductive organs of *L. bostrychophila*. Little or no data exist on psocid gross anatomy, organisation and the ultrastructure of their internal organs.

The ovaries are of the polytrophic meroistic type, which is characterised by the presence of a cluster of nurse cells forming part of the growing egg chamber. In the early stages of oocyte development it is difficult to distinguish between cells that will differentiate to become an oocyte or nurse cells. These nurse cells produce nearly all of the RNA of the egg and ultimately that of the ooplasm through the fusion of the nurse cell cytoplasm with the egg (Mahowald, 1972). This type of ovary is found in a wide range of insect orders (King and Buning, 1985; Gerber *et.al.*, 1978). In some insects the polytrophic ovarioles produces a series of eggs at one time. Insect orders with this type of ovary include Diptera, Hymenoptera, and Lepidoptera. This mode of egg formation allows for rapid and continuous egg laying. The newly formed oocytes of the fruitfly *Drosophila melanogaster* for example, under optimal condition can undergo a 100,000-fold increase in volume in three days, and can produce a quantity of eggs equivalent to 30x the body weight in 10 weeks (King, 1970). *L. bostrychophila* in optimum conditions can produce the body weight in egg mass in one day and up to 25x in the first 7 week of egg production.

The reproduction system of the booklouse *L. bostrychophila* consist of a pair of ovaries branching from the common oviduct and running almost parallel to each other and to the axis of the female abdomen. In insects the number of ovarioles varies but are species-specific (King and Buning, 1985). Some species of Scarabaeini have only one ovariole (Crowson, 1981), Whilst there may be as many as 1000 ovarioles in the blister beetle *Meloe proscarabaeus* (Buning, 1979). In *L. bostrychophila* each ovary contains 5 ovarioles.

Buning and Sohst (1990) suggested that the psocid ovary represent the basic model for polytrophic meroistic ovary structure in insects, based on their study of *Peripsocus phaeopterus* and *Stenopsocus stigmaticus*.

How the number of ovarioles in the psocids is related to egg production (specifically those studied, *Peripsocus phaeopterus* (8), *Stenopsocus stigmaticus* (10) and *L. bostrychophila* (10)) is a moot point. Increased number of ovarioles may be expected to be limited to a greater egg production or may be simply a function of body size. However L. *bostrychophila* is considerably smaller then *P. phaeopterus* and *S. stigmaticus* and there is little variety in ovariole number.

Ries (1932) first described the polytrophic meroistic ovaries of the Phthiraptera. The recent reinvestigation of the morphology and the ultrastructure of the ovary of the bird lice (Mallophaga) by Bilinski and Jankowska (1987) and Bilinski (1989) added weight to the phylogenetical origin of this type of polytrophic meroistic ovary. Results obtained from *L. bostrychophila* in this study, as well as the investigation on the ultrastructure and cell cluster analysis of feather louse and that of two species of bark lice (Psocoptera) by Buning and Sohst (1988; 1990), provide additional evidence that the phylogeny of the hemipteroid group of insects is founded on a psocodean ancestor.

Each ovariole of *L. bostrychophila* is divided into small units (anterior-posterior position). The structure and the constituents of these ovarial units are described. The most anterior or upper portion of the ovariole (the germarium) lie the early growth stages containing the daughter cells. One of these cells will differentiate into an oocyte and the rest into nurse cells. The number of the nurse cells is constant within each species, in *L. bostrychophila* (*N=3*). In the sucking louse *Anisolabis maritima* (Anoplura) with similar type of ovaries to that of psocids, the number of nurse cells is reduced to one, while in some species of Hymenoptera the oocyte is associated with up to 31 nurse cells (Bonhag, 1956).

When the oocyte is approximately one-half the size of the mature egg, the nurse cells break down and their cytoplasm pours into the oocyte. This doubling of the size at the end of oogenesis in *L. bostrychophila* is insignificant compared with the 100,000-fold increase that occurs at this stage of development in the fruitfly *Drosophila melanogaster* (King *et.al.*, 1956). Follicle cells are of high synthetic activity as they are the site of the production of yolk material in most insects and synthesise subsequent layers around the egg at the end of vitellogenesis (Buning, 1994). The ultrastructural evidence in *L. bostrychophila* showed pronounced nuclei of the follicular cells in previtellogenic and

vitellogenic stages of the developing oocyte. There are gaps in the follicle border between the follicle cells and the oocyte, in contrast to the outer layer of the follicles, which is continuous electron dense membrane. These gaps between oocyte and follicle cells are thought to give passage to egg bound vitellogenins by pinocytotis during vitellogenesis (Telfer *et.al.*, 1982).

Two patterns of contact between follicle cells and oocyte have been observed. The first type is where the junction is convoluted and is observed in the bugs (Huebner and Injeyan, 1981), moth ovaries (Woodruff, 1979), *Hylophora* and *Rhodnius* (Telfer *et.al.*, 1982) as well as other insects (Munz, 1988). The follicle invaginations are thought to be ion current pathways and speed oocyte development (Munz, 1988).

L. bostrychophila is an example of the second type where the follicle cell/oocyte border remains smooth throughout the oocyte development, a situation also seen in the bird louse (Blinski and Jankowska, 1987), curculionid beetles (Bilinski and Petryzak, 1978) and whirligig beetles (Bilinski and Szklarzewicz, 1987).

The structure and thickness of follicle cells varies from species to species (King and Burnet, 1959; Buning, 1994). Follicle cells are symmetrically arranged around the mature oocyte of *L. bostrychophila* and are the site of vitellogenesis. The EM micrographs of vitellogenic oocytes show small globules of yolky material inside the follicle cells and released into the oocyte. These droplets form large globules of yolk material inside the oocyte. In the cytoplasm of a healthy liposcelid oocyte the globules comprise the most of the volume of the egg, however, in infected oocytes the yolk material is a fraction of that of the normal oocyte.

The vitelline membrane is elastic with finger-like infoldings within the ooplasm measuring up to 21μm deep. Such infoldings have been recorded in the micropylar part of the vitelline membrane in Coleoptera (Sweeny *et.al.*, 1968), Lepidoptera, Drosophilidae and other insects (Margaritis, 1985). The infolding of the vitelline membrane and subsequent formation of micropylar structure plays an important role in the sperm-oocyte interaction as point of sperm entry (Gerrity *et.al.*, 1967; Sweeny et.al., 1968). Most insect species reproduce sexually; since *L. bostrychophila* is completely parthenogenetic, the structure of the mature egg and the presence of a non-functional spermatheca indicate that parthenogenesis has arisen more recently.

CHAPTER THREE

THE RICKETTSIAL ENDOSYMBIONTS OF *L. BOSTRYCHOPHILA*

CHAPTER THREE

3.0. THE RICKETTSIAL ENDOSYMBIONTS OF *L. BOSTRYCHOPHILA*

3.1. INTRODUCTION

This chapter describes the morphology, distribution and the identity of *Rickettsia*-like organisms (RLO) found in the ovaries of *L. bostrychophila.* Investigations of these endosymbionts were conducted using a variety of approaches, including light microscopy, EM and culture of the bacteria in a cell-free medium.

Intracellular bacteria were first reported within the reproductive tissues of mosquitoes *Culex pipientis* by Hertig and Wolbach (1924). The RLO were subsequently placed in the genus *Wolbachia (W. pipientis)* some 60 years ago by Hertig (1936). Since then many insect species have been found to be infected with a variety of microbial agents, mainly rickettsiae. Steinhaus (1947) has given a comprehensive account of known microbes associated with insects and ticks. Lysenko (1963) and Buchner (1960) provided the current basis for the classification of entomogenous bacteria using physiological and serological characteristics. Recently genetic properties, as well as the pathogenicity to the insect host have been used (O'Neill *et.al.*, 1997).

Bacterial endosymbionts can be found in different parts of the body. In the majority of cases, sex altering intracellular bacteria are found in the reproductive organs (Brinton, 1969). *Rickettsia* and other intracellular bacteria are also found in the fat bodies, salivary glands (Kokwaro, *et.al,* 1990) malpighian tubes (Burgdorfer, *et. al.*, 1973), or other specialised organs and structures like the mycetocytes (Musgrave *et.al.*, 1962). The information on the location, identity, mode of transmission and function of intracellular bacteria has been reviewed by Steinhaus, 1947; Richard and Brooks (1958); Koch (1960, 1967), Buchner (1965); Francke-Grosmann (1967); Douglas (1989, 1994).

Their known impact on the reproductive system of other insects has been summarised by Giordano *et.al.*, 1995; Hurst *et.al.*, 1996; Chang and Wade 1994, 1996; Werren 1993, 1997. Recently there has been an upsurge of interest in these microrganisms since they are implicated as causing, among other things, cytoplasmic incompatibility (Yen and

Barr, 1971; Breeuwer *et.al.*, 1993, 1996) and the induction of parthenogenesis (Stouthamer *et.al.*, 1993, 1994; Stouthamer and Werren, 1993).

3.1.1. Rickettsial transmission mechanisms

Special transmission mechanisms have evolved to ensure the transfer of the endosymbionts to the next generation. In some insects symbiont filled capsules are laid among the aposymbiotic egg masses and the hatching nymphs receive the symbionts by drinking the bacteria-containing fluid of the capsule (Schneider, 1940). In other cases, special organs smear the endosymbionts on to the external surface of the egg at the time of oviposition, and the nymphs acquire the infection either by eating the eggshell (Breitsprecher, 1928), or via the micropyle before being laid (Douglas, 1994). A more intimate mode of transmission however, is that observed in this study where the symbionts enter the egg during early oogenesis of the developing oocytes in the ovarioles. Bacteria with such complicated transmission mechanisms are known to be difficult to eliminate experimentally from the insect tissues. Surface sterilisation and cleaning of insect's eggs therefore does not affect the transmission of the bacteria from mother to progeny (Pant and Dang, 1972).

3.1.2. *Rickettsia*-like organisms in psocid systems

Although some morphological investigations have been carried out on the internal organ system and anatomy of various psocid species (Badonnel, 1934; Goss, 1952, 1954; Wong and Thornton, 1966), they have all failed to record any endosymbionts in the tissues and organs they have investigated. Two interesting accounts exist in early literature whereby rickettsiae were seen in a "dust louse" *Psocus* sp. in the family Psocidae (Sikora, 1918) and in the "book louse" *Dorypteryx pallida* in the family Atropidae (Hertig and Wolbach, 1924).

In the case of *Psocus* (Sikora, 1918) reported that rickettsiae were observed on the stomach epithelium, every individual was infected and transmission was assumed to be via the egg stage.

Interpretational problems associated with these early records in psocids are discussed by Yusuf *et.al.*, (1999). *Psocus* is a generally applied generic name for the psocids but is not normally used for dust or book lice leading to doubt as to which species was studied.

In the present study the *Rickettsia*-like organisms were not found to be associated with gut. *Dorypteryx pallida* (Atropidae) is a bisexual rather than a parthenogenetic species; bisexual liposcelids that have been tested (*L. corrodens* and *L. entomophila*) do not contain such inclusions.

3.1.3. Methodologies used to study rickettsiae in psocid tissues.

A variety of methods were used in studying the intracellular rickettsiae depending on the level of detail required. The electron microscope was used when detail of the ultrastructure were needed. However when a more rapid survey for the presence of bacteria in the psocids was required, temporary mounts of the ovaries were examined using light or fluorescent microscopy.

A variety of staining techniques recommended for the demonstration of rickettsiae from insect tissues were tried. These included Machiavello's (Gridley, 1960; Machiavello, 1937), Giemsa's (Hertig, 1936) and Gimenez's techniques (Gimenez, 1964). These methods all stained DNA in the rickettsiae, highlighting them against the DNA free host cytoplasm. Fluorescent stains binding to the rickettsial DNA have been used in the diagnosis and the abundance estimates of these parasitic bacteria (O'Neill and Karr, 1990; Boyle *et.al.*, 1993). Transmission electron microscopy is also universally applied to describe the presence and the ultrastructure of rickettsiae in arthropod tissues. The ultrastructural details of the endosymbionts are taxonomically important especially when dealing with the polymorphic rickettsiae (Wright *et.al.*, 1980).

The advantage of using light microscopy over the EM as the routine method for detecting rickettsial infections was the ease with which a large number of samples could be quickly screened. The preference for the use of fluorescent staining lies in the sensitivity and rapidity of diagnosis.

3.2. MATERIAL AND METHODS

Both adults and eggs were used to provide microscopic material. To avoid potential bacterial contamination both eggs and adults were surface sterilised before dissection.

3.2.1. Surface sterilisation of adults

Adults were surface sterilised using a succession of three washes of 5ml 70% ethanol for 1 min interspersed by sterile distilled water washes for 1 minute each.

3.2.2. Surface sterilisation of eggs

The surface of the egg was sterilised in an attempt to remove any surface inhabiting bacteria using the method of Norris, 1972. The surface sterilisation technique did not interfere with the viability of eggs. Eggs were laid on filter paper and removed by teasing them off with fine brush. Psocid eggs are soft and turgid; therefore they are easily burst. Eggs were washed in distilled sterile water for 1 minute, and then submerged in 0.1% $HgCl_2$ for 4 minutes. The eggs were then rinsed with two 1-minute washes of distilled water followed by 30 seconds in 70% ethanol, and finally rinsed with distilled, sterile water for 1 minute. The eggs were transferred to an aseptically cleaned surface (slide) for further processing e.g. squash preparation, fixation for EM analysis or smeared on the surface of a serum artificial medium to culture.

3.2.3. Dissection of adults

The surface sterilised psocids were dissected in sterile 0.8 % sodium chloride or Ringer's solution. A single female psocid, placed in the depression of a cleaned cavity microscope slide, was covered with a drop of sterile *Drosophila* Ringers solution. Grasping the thorax firmly using forceps, the abdomen is opened laterally using a pin from the mesopleuron toward the ovipositor and the reproductive system removed. Dry smears of the fresh tissue preparations were stained and examined with phase contrast microscopy. Similarly prepared fresh psocid tissues were also stained for fluorescent microscopy.

3.2.4. Staining and light microscopy detection assay I:

The following procedure to detect rickettsiae was reported to produce a reproducible results and is used in this study (Nogge *et.al.*, 1981): Ovaries were dissected in Belar's saline[1] (Breland, 1961) and transferred with a needle to a clean microscope slide, smeared and dried. The tissue was fixed with absolute methanol for at least 10 minutes. The ovary smear was then stained with Giemsa in 1:50 dilution. The pH was maintained between 7.2 - 7.4 using freshly prepared phosphate buffer. The smear is left in the stain for 2 hours. Stained smears were rinsed in distilled water and gently blotted or air-dried. The stained smears were examined for the presence of bacteria with oil immersion optics.

Of the staining methods tried that of the Machiavello proved the most successful. To confirm that the smears are *Rickettsia*-positive, Machiavello's (1938) method uses a thin smear preparation of tissues and is universal for staining rickettsiae. The ovarial tissues were either air-dried or the slide warmed gently over a Bunsen burner. The smears were then stained for 4 minutes in a filtered aqueous solution of 0.25% acid fuchsin adjusted to pH 7.5 with phosphate buffer. The stained tissue was rapidly washed with 0.5% citric acid, followed by tap water and finally counter stained with methylene blue. This process stains the rickettsiae red against a blue background of the psocid tissues.

3.2.5. Fluorescent microscopy detection assay II

An alternative approach using a fluorescent stain that binds to the rickettsial DNA provided a simpler and more rapid method to visualise the presence of bacteria in the ovaries of *L. bostrychophila.* The reproductive organs of surface sterilised adults, dissected as previously outlined, were placed in fresh Ringer's. The Ringer's solution was then removed with paper tissue and replaced with 4µl of (2.5 mg/ml) stock Hoechst 33258 (Sigma) solution diluted in 10ml of Drosophila ringers. The tissue was incubated in the staining solution at room temperature for 10 min. The tissues were then transferred

[1]**Belars Saline: Sodium chloride, 6.1gm.; Potasium chloride, 0.2gm.; Calcium chloride, 0.2gm.; Sodium bicarbonate, 0.2gm. Distilled water is added to make 1 litre of Belars saline.**

to fresh Ringers solution and incubated for a further 10 minutes. The ringers solution was then drawn off using paper tissue and immediately replaced with a drop of sterile glycerol for observation under fluorescent microscope. The fluorescent staining technique is also useful in quantifying the amount and the densities of the bacteria in the infected insect tissues. This will be explored in chapter six.

3.2.6 Electron microscope detection assay III:

Transmission electron microscopy gives the most detailed information on the presence of rickettsiae in psocid tissues. Histological methods employed here are similar to those described earlier. Adults were killed, fixed, and embedded following the methods outlined in chapter two. The polymerised resin blocks were sectioned and structures bearing rickettsial cells examined using a Jeol 100 KII TEM.

3.2.7 Attempts in culturing *Rickettsia*-like organisms from psocid gonads.

It would be valuable to be able to culture these bacteria, however this is extremely difficult. The only successful attempt at culturing bacteria in the tribe *Wolbachiae* was with *Wolbachia persica*. This is a non-pathogenic rickettsia isolated from the ovarial tissue of the tick, *Dermacentor andersoni,* and injected into chick embryos for cultivation (Burgdorfer *et. al.*, 1973).

Attempts were made to culture bacteria from infected individuals. Squashes of reproductive organs from surface sterilised *L. bostrychophila* adults and eggs were prepared. The squashes were directly placed on inactivated bovine serum (Gibco™) in 60 x 15 mm tissue culture dishes. This serum based medium is similar to that used for other fastidious organisms (e.g. Mycoplasma). Inoculations were carried out in aseptic conditions in a laminar flow hood. Petri dishes were placed in a large plastic box, tightly sealed and incubated in various temperature regimes (20°C, 25°C, 30°C) corresponding to the temperature of natural habitat of the insect. The Petri dishes were kept in this condition for 3 days or until colonies of bacteria were observed. Gram stained smears were made from any bacterial growth observed and the presence of small coccoid bacteria

with the characteristics of rickettsiae searched for. However this proved unsuccessful and will not be discussed further.

3.3. RESULTS

3.3.1. Light microscope

The results obtained from light microscopy were generally disappointing. Using simple stains and observing the rickettsial inclusions under the light microscopy produced many difficulties. Reproducibility of results obtained from one stain, or between different stains varied in an unpredictable manner. Other difficulties included distinguishing rickettsial inclusions from cell granules and other artefacts. In Giemsa stained tissues for example, granules, precipitates of the dye and other artefacts commonly occurred which made it difficult to distinguish the bacteria. *Rickettsia*-like organisms infecting the reproductive tissues of *L. bostrychophila* were highly polymorphic, a characteristic shared by all intracellular Wolbachiae. This contributed to the difficulties in examining *Rickettsia* with light microscopy. These techniques, however, were useful in determining when large masses of tiny *Rickettsia*-like organisms were present in the ovaries of *L. bostrychophila*, but not in the ovaries of uninfected bisexual liposcelids (*L. corrodens, L. entomophila*). Permanent preparations of the ovaries of healthy bisexual liposcelids and infected ones from *L. bostrychophila* were made and used for comparison.

The rickettsiae were successfully imaged in Giemsa stained smear preparations of the ovaries of *L. bostrychophila,* but the best results were obtained using Machiavello's stain on ovarial squashes. The rickettsiae appeared as brightly coloured red spots in a blue background (Plate 3.1).

3.3.2. Fluorescent microscope staining

The flourochrome (Hoechst 33258) stained ovary preparations revealed the bacteria as aggregations of highly luminescent particles against faint background fluorescence concentrated on the peripheries of the tissues (Plate 3.2). The flourochrome binds with

DNA in the bacteria creating a bright glow under fluorescence microscopy where the bacteria were clustered.

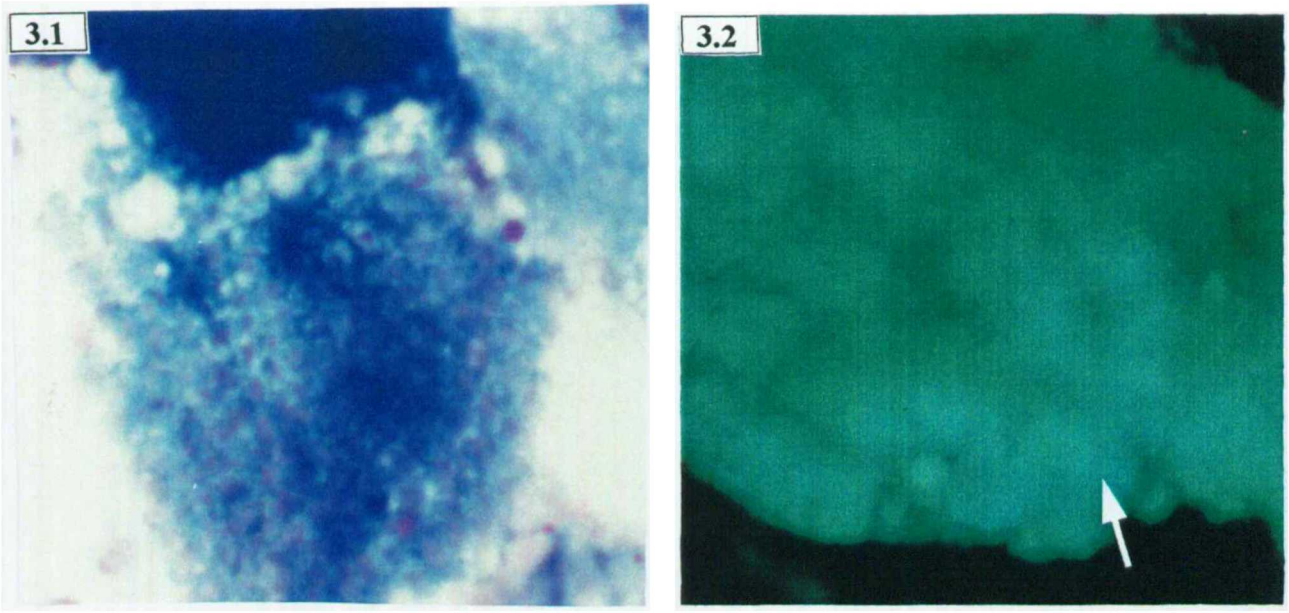


Plate 3.1 Machiavello staining of rickettsiae from *L. bostrychophila* ovaries. Rickettsiae are shown as very small brightly coloured red spots against a blue background of the psocid tissues. Magnification 2500x.

Plate 3.2. DNA-positive bodies of *Rickettsia* infected ovarial tissues stained with Flourochrome (Hoechst 33258) and observed with fluorescent microscope. Rickettsial inclusions are shown as highly fluorescent bodies in the peripheries of the ovarial tissues (arrowed). Magnification 500x.

This method is rapid in detecting the presence of *Rickettsia* in the psocid ovaries. The ovaries of bisexual liposcelids processed in a similar fashion, reveal no fluorescent bacterial bodies and show a less intense, uniform background fluorescence. This reduced emission of fluorescence per unit area in healthy bisexuals is translated in to a higher exposure time registered in the photographic exposure meter connected to the epiflorescent microscope (Plate 4.10-11, Chapter 4).

3.3.3. Electron microscopy

Sections of the abdomen of *L. bostrychophila* were thoroughly examined under the electron microscope and bacterial endosymbionts were found sub-cuticularly and concentrated in the oocytes.

3.3.3.1 Ovarial infection in *L. bostrychophila*

Each oocyte cytoplasm contains a diversity of inclusions including electron dense spherical bodies, yolk and lipid globules and various other organelles. The cytoplasm matrix is organised in a mosaic fashion as revealed in sections with much of the matrix showing a fine granular appearance interspersed with "islands" of less electron dense material. Within these lighter islands large numbers of bacterial cells were found (Plate 3.3). These inclusions were identified as bacteria because of the presence of a double cell wall. This cell wall is approximately 15-20nm thick and surrounds the dense bacterial protoplast but is separated from it by a clear, periplasmic space up to 75nm wide in places. The bacteria vary in size and shape from coccoids (0.18-0.25μm in diameter) to bacilliforms (0.25x1.5μm), with an outer cell wall, inner plasma membrane, and a cytoplasm of ribosomes and filamentous DNA strands (Plate 3.4). The size of the bacterial cells varies according to their developmental stages, but in general they are comparatively small. The mature vegetative stages grow to short round-ended cylinders and sausage-shaped form (Plate 3.5). These later reproduce by means of binary fusion, when two cells, each more or less rounded in shape, are produced (Plate 3.6). These newly formed cells shrink and condense as they undergo further development and soon establish the internally occluded appearance typical of mature rickettsiae.

The bacterial cells are not indiscriminately scattered throughout the insect ovary but are arranged in clusters. Clusters occurred only in the oocyte cytoplasm, and the bacteria were not observed in the follicle cells, muscular layers or any other cell type in the ovary. Every *L. bostrychophila* oocyte (approximately 150) which has been examined with transmission electron microscopy has contained these bacterial cells. Extrapolations from the number of bacterial cell profiles visible in a single longitudinal section through an oocyte suggest that the total bacterial complement of each oocyte must be considerably in excess of 1000 cells.

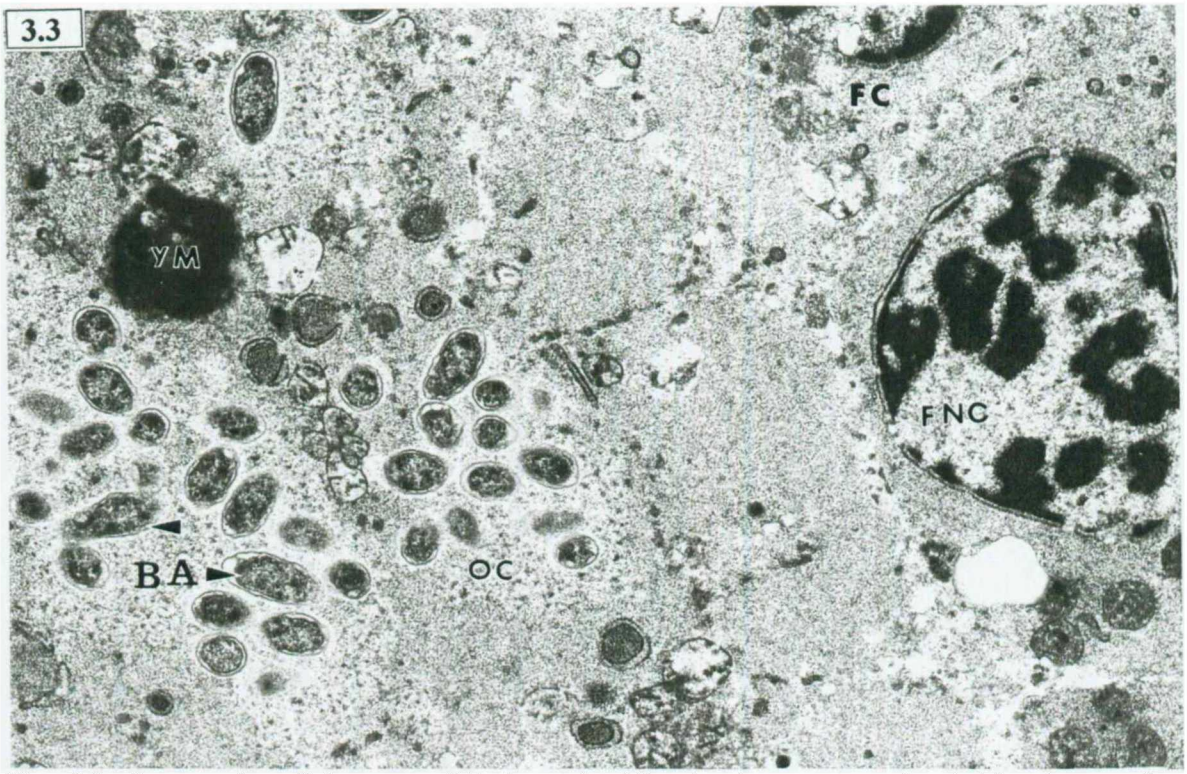


Plate 3.3. Cross section of the ovary of *L. bostrychophila*, showing an oocyte invaded by clusters of coccoid rickettsial cells. FC, Follicle Cells; FNC, Follicle nucleic cells. OC, Oocyte; YM, Yolk material; BA, Bacteria. Magnification 14400x.

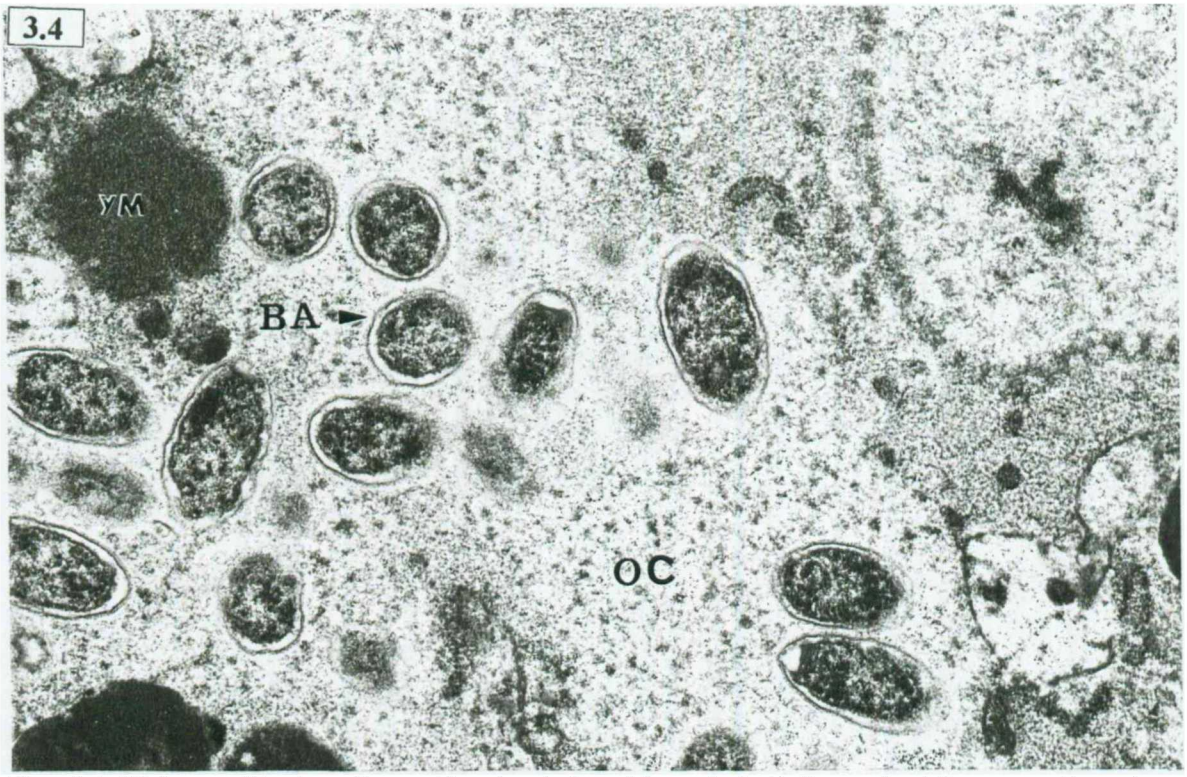


Plate 3.4. High magnification of coccoid and rod shaped bacteria in *L. bostrychophila* oocyte. Bacteria with double wall but without any surrounding host cell membrane. BA, Bacteria; OC, Oocyte; YM, Yolk material. Magnification 28000x.

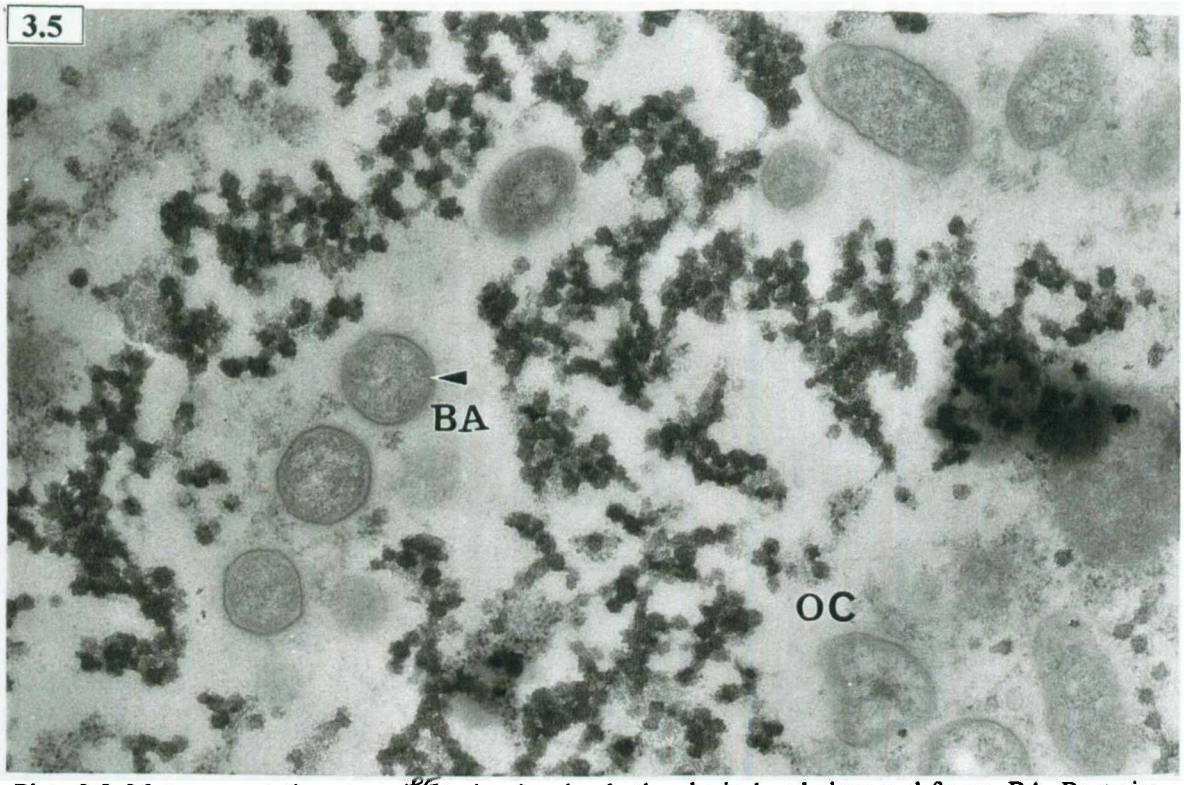


Plate 3.5. Mature vegetative stages of bacteria showing both spherical and elongated forms. BA, Bacteria; OC, Oocyte cytoplasm. Magnification 28000x.

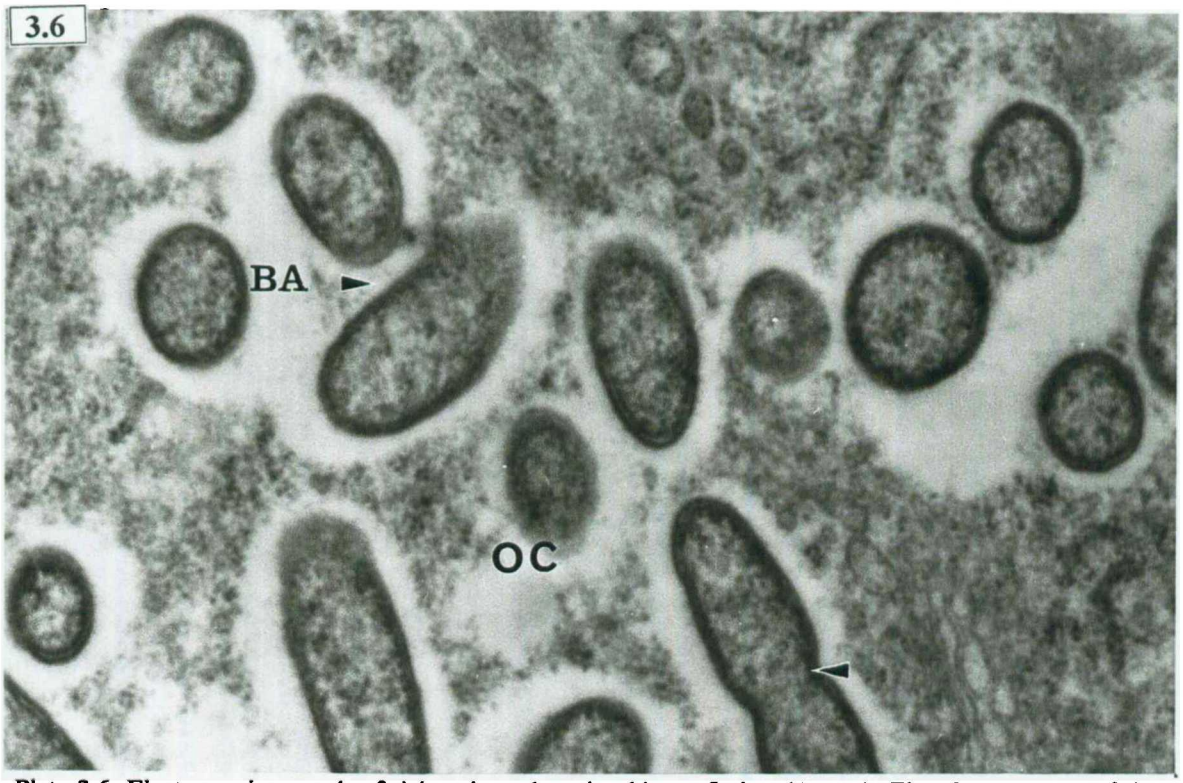


Plate 3.6. Electron micrograph of rickettsia undergoing binary fission (Arrow). The ultrastructure of the cytoplasm changes with the presence of the bacteria. Bacterial cells are always surrounded by a less electron dense area. BA, Bacteria; OC, Oocyte cytoplasm. Magnification 38000x.

Bacterial cells are always surrounded by a less electron dense area measuring approximately 0.3μm (Plate 3.5-6). This spatial co-occurrence of the bacterial cells and the lighter island could be taken as conclusive evidence that the changed cytoplasmic appearance is linked to the presence of the bacteria. The clear zone separating the organisms from the cytoplasm may be an area of lysis. The highest magnification of one of such bacterial cells shows an ultrastructure consisting of an outer granulating cortical zone and an inner, less dense, vacillated zone (Plate 3.7). There is no apparent limiting membrane between the two zones. The bacteria may be sparsely or densely distributed throughout the cytoplasm of the oocyte (Plate 3.8). Microrganisms were usually seen singly within any given cluster, despite tight spacing between them.

Density of the rickettsiae varies in two ways. As an oocyte develops so the bacterial density increases, and as a female ages so her oocytes contain a higher density of the bacteria (Plate 3.9).

The morphological resemblance of these inclusions to known rickettsiae is striking. No host membrane appear to surround these bacteria, instead they are dispersed in the host cytoplasm like most other parasitic rickettsiae. The ultrastructure of the endosymbionts isolated from the parthenogenetic psocid is identical to that of *Wolbachia pipientis*. This species is obligate intracytoplasmic, transovarially-transmitted symbionts of insects and the type species of the genus *Wolbachia* in the Rickettsiales.

Based on these morphological features, the rickettsia found is tentatively identified as *Wolbachia*-like. The genus *Wolbachia* has been frequently identified in the reproductive tissue (ovaries and testis) of the arthropods.

These findings suggest that the association between the rickettsiae and the psocid host is an intimate one. Electron microscope sections of the eggs, juveniles, and ovipositing adults of *L. bostrychophila* all reveal the presence of the rickettsiae which indicated that transmission of the bacteria from mother to progeny is mediated via the egg. In *L. bostrychophila* the infection appears to be established in the oocyte in early oogenesis.

The number of bacterial cells increases in the oocyte cytoplasm as the psocid gonad develops. No simple mechanical or chemical treatment of the exterior of these eggs was found to result in bacteria-free offspring.

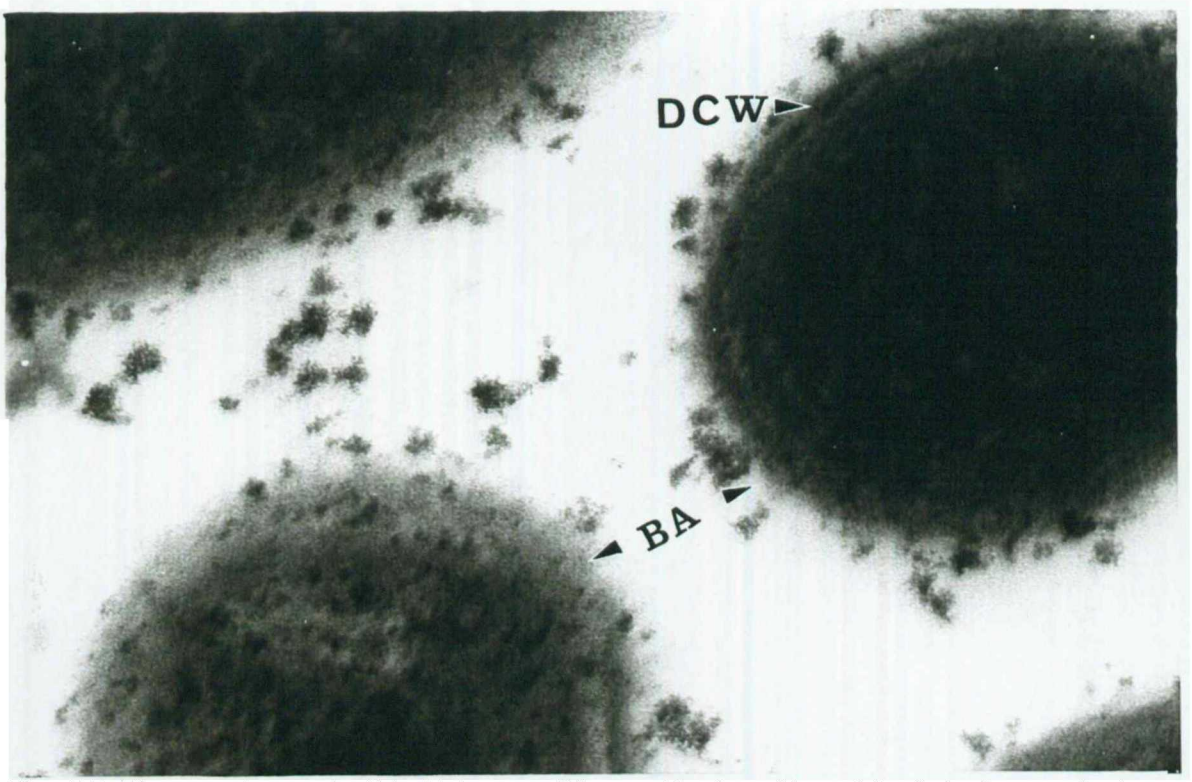


Plate 3.7. Electron micrograph of the highest possible magnification of bacterial cells in the reproductive tissues of the *L. bostrychophila.* BA, Bacteria; DCW, Double cell wall (Bacteria).Magnification 200000x.

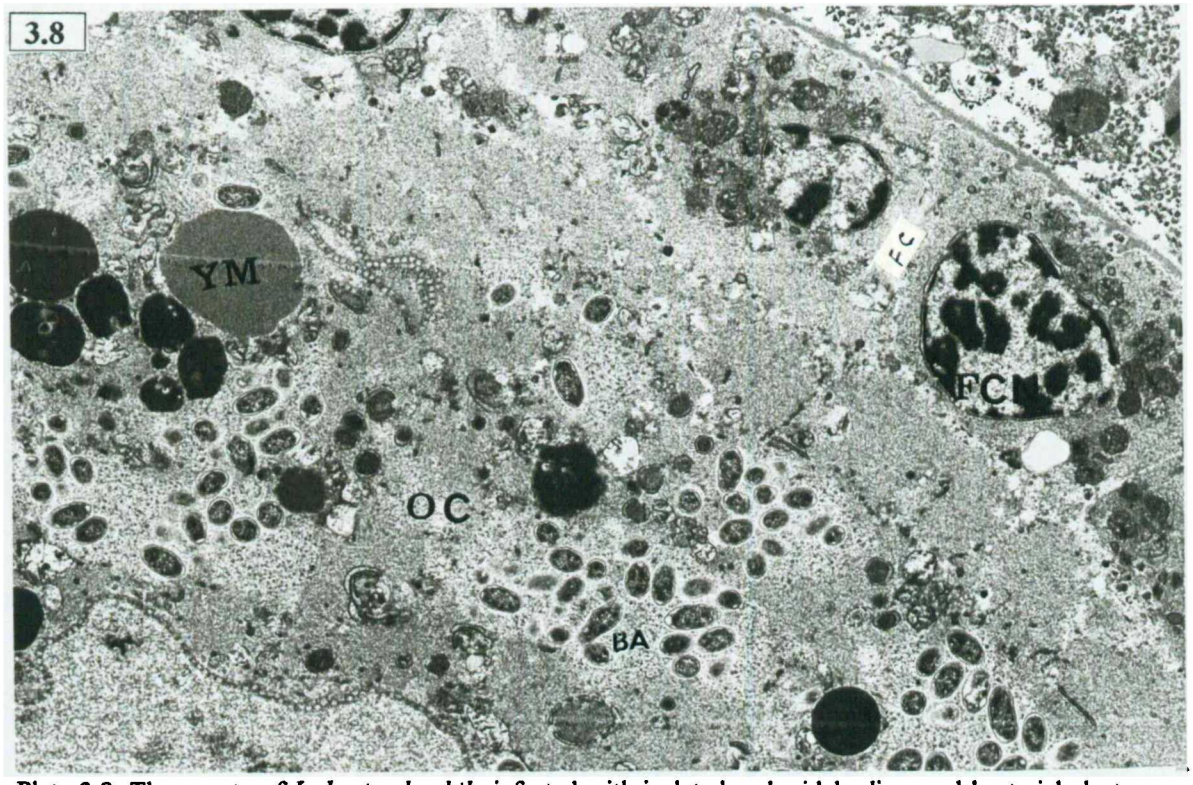


Plate 3.8. The oocyte of *L. bostrychophila* infected with isolated and widely dispersed bacterial clusters. FC, Follicle Cells; FCN, Follicle nucleic cells. OC, Oocyte; YM, Yolk Material; Ba, Bacteria. Magnification 7200x.

Plate 3.9. Oocyte section of an old *L. bostrychophila* with a high density of bacterial cells. Heavily infected ovaries shows some sign of degeneration. BA, Bacteria; VA, Vacuoles. Magnification 28000x.

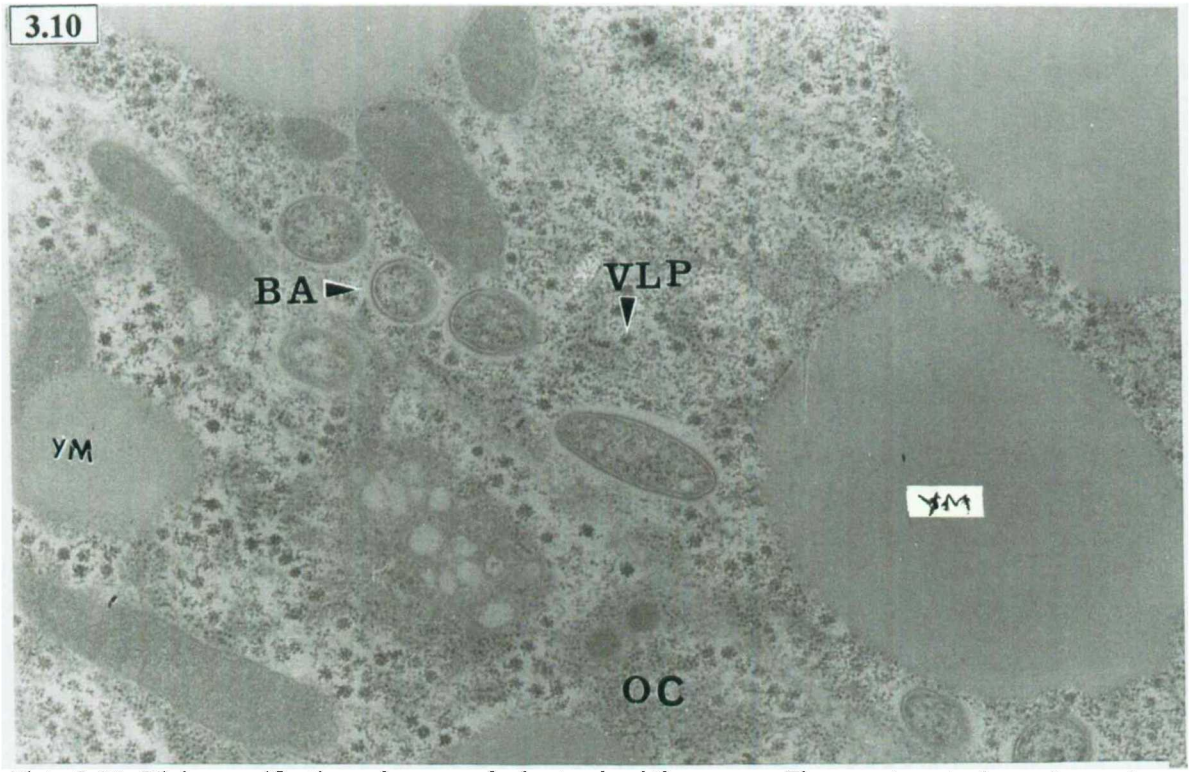


Plate 3.10. High magnification of mature *L. bostrychophila* oocyte. The oocyte cytoplasm shows the presence of bacterial cells surrounded by virus-like, electron dense particles. BA, Bacteria; VLP, Virus-like particles; YM, Yolk material; OC, Oocyte cytoplasm. Magnification 20000x.

Although the bacteria are abundant in the oocytes they were not seen in the follicle cells of *L. bostrychophila* (Plate 3.11). However, they were occasionally found outside the reproductive system in the Sub-epidermal region in the body cavity of the adult abdomen (Plate 3.12). How the rickettsiae get from the subcutaneous region to the oocyte is unknown.

Associated with the rickettsial clusters in the cytoplasm of some oocytes are inclusions of small, electron dense virus-like particles (Plate 3.10). These have a rounded structure with diameter of 20nm. Nothing more is known of these particles.

3.3.4. Polymorphism of the rickettsial endosymbionts of *L. bostrychophila*

Bacterial cells from *L. bostrychophila* tissues observed under the transmission electron microscope exhibit a variety of shapes. The most frequently encountered structure is the coccoid shape, measuring 0.14-0.16μm in diameter (Plate 3.3). In some bacteria spherical or polyhedral inclusions were seen (Plate 3.13). Their cytoplasm consists of a granular outer cell wall (the cortical region) in which densely staining DNA threads are aggregated along the inner surface of the cell envelope. The DNA strands are always peripherally oriented and the centre areas of the bacterial cells are electron-translucent (Plate 3.14-15).

Other elongated and narrow forms of the endosymbionts measuring 1.5μm were also observed (Plate 3.15). Occasionally their length reaches 3.1μm. The ultrastructure of this elongated form is far less electron dense. The cell content is more granular and dispersed.

Less frequently encountered are endosymbionts with a tail-like structure 0.83μm long by 0.17μm wide. This form mainly occurs in the body cavities among other bacterial structures. The ultrastructure of this form is more electron dense (Plate 3.16).

The space between the outer cortical zone and the inner wall in some bacterial cells is not always clear. This might have been caused by the fixative used. Frequently the bacterial cells are visible as electron dense rounded bodies in transverse section.

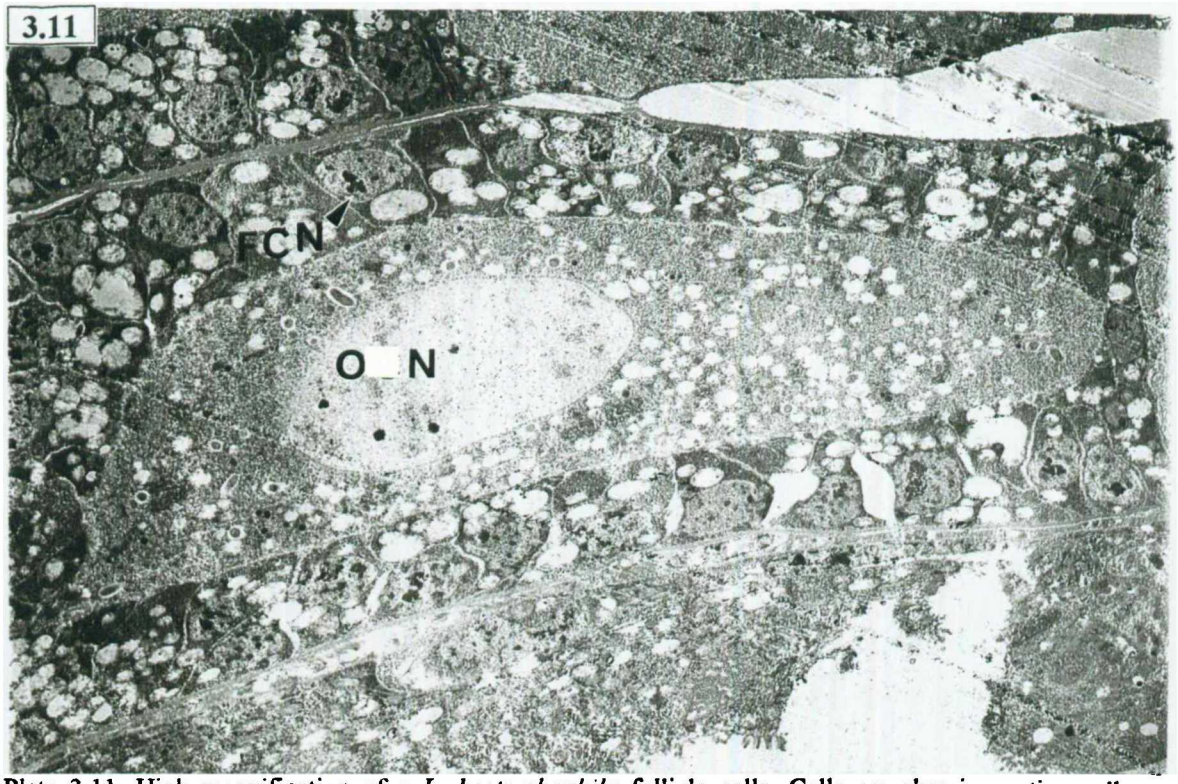


Plate 3.11. High magnification of a *L. bostrychophila* follicle cells. Cells are showing active cell division. Follicle cells of *L. bostrychophila* have no bacterial infection. FCN, Follicle cell nucleus; O N, Oocyte nucleus. Magnification 2800x.

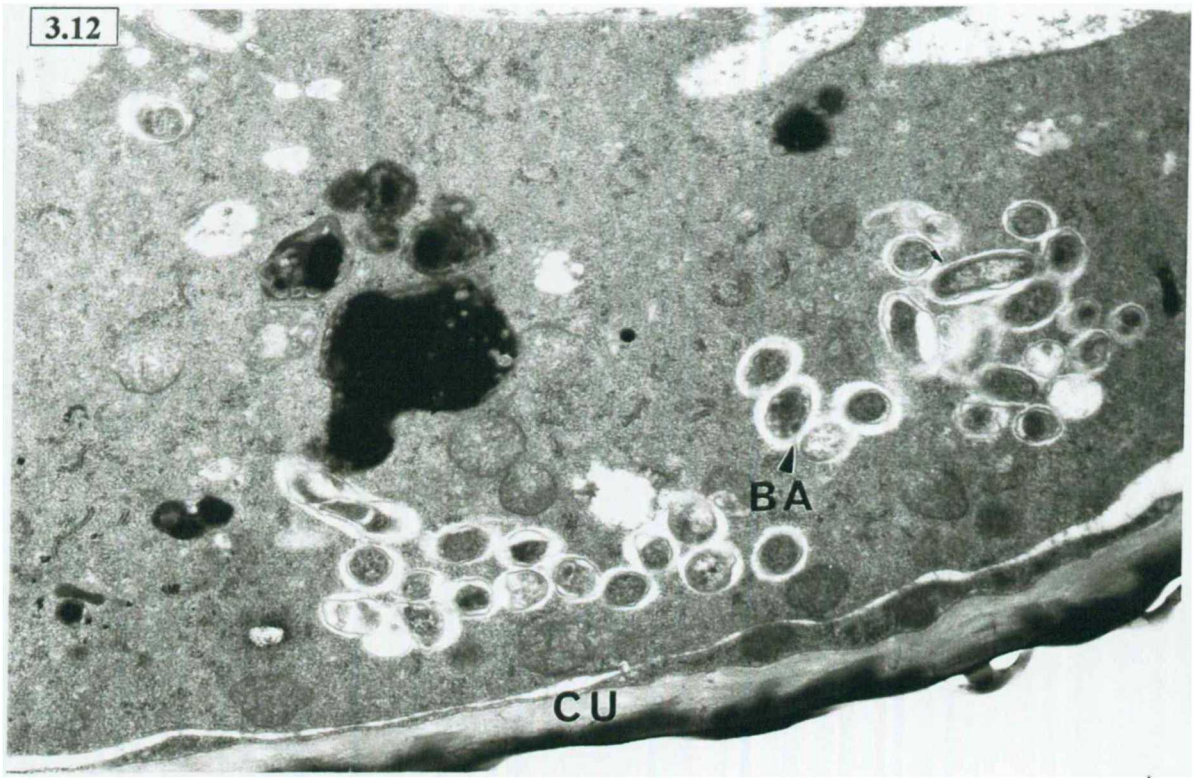


Plate 3.12. Rickettsial infection of sub-cutaneous body cavity of *L. bostrychophila*. B Bacteria; CU, Cuticle; Magnification 14400x.

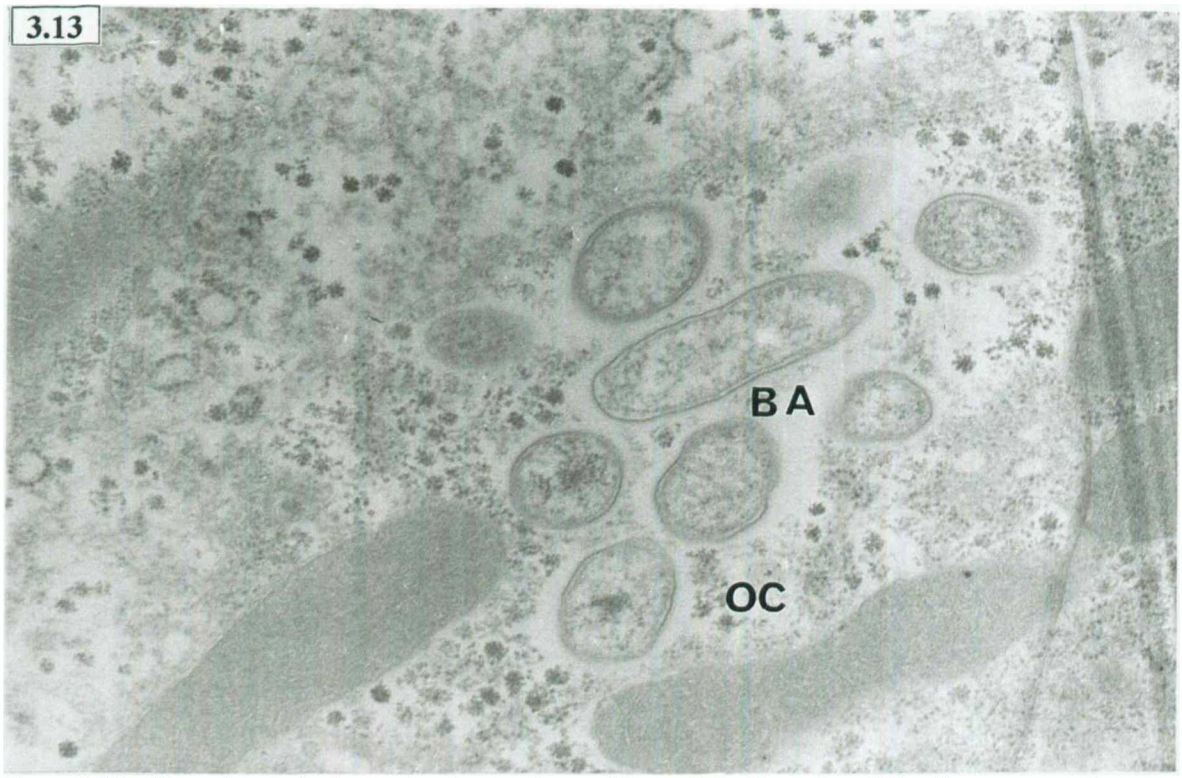


Plate 3.13. Bacterial inclusions showing polymorphism. BA, Bacteria; OC, Oocyte cytoplasm. Magnification 28000x

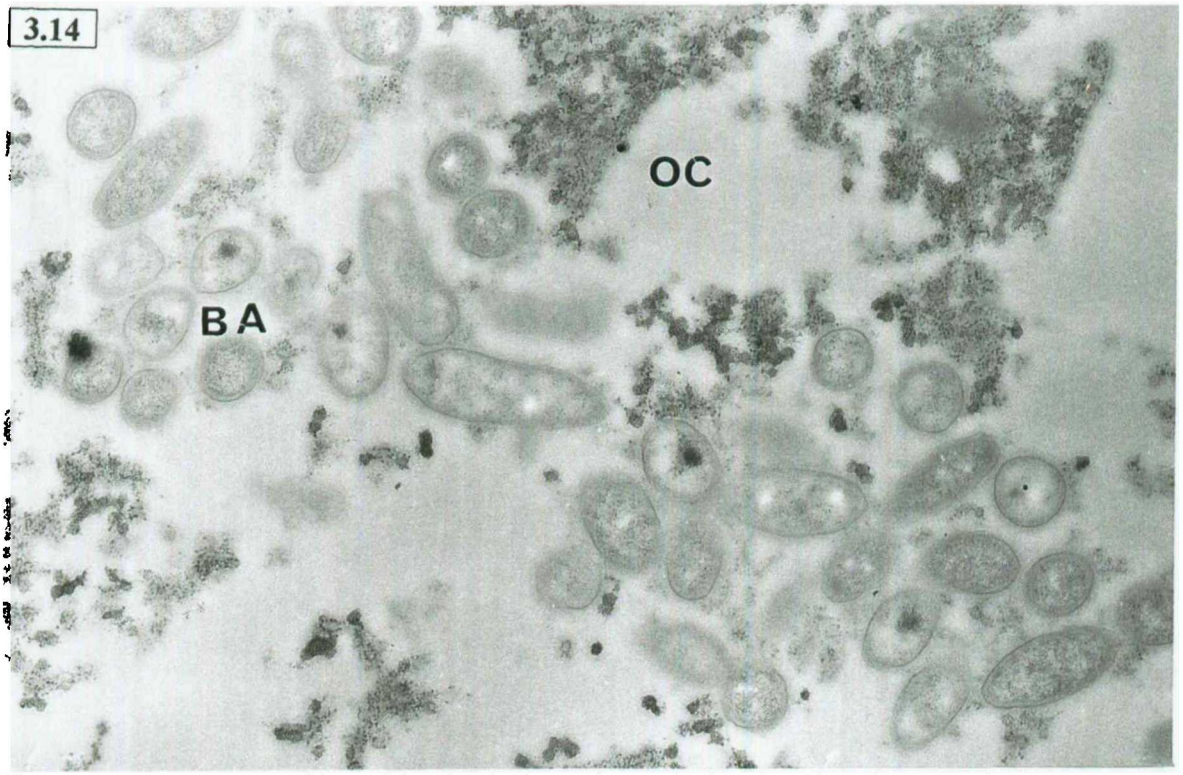


Plate 3.14. Ultrastructure of bacterial cells, the internal material is oriented at the cell periphery with an electron-translucent centre. BA, Bacteria; OC, Oocyte cytoplasm. Magnification 20000x

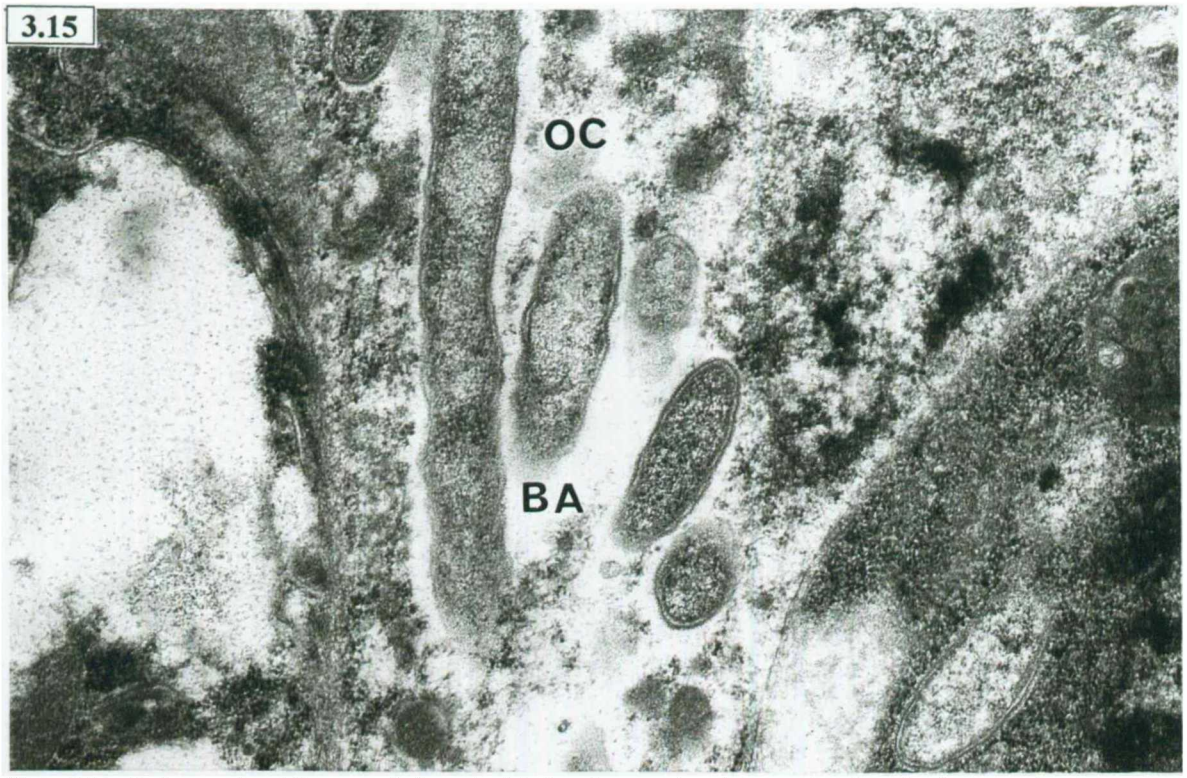


Plate 3.15. *L. bostrychophila* ovary infected with the elongated and coccoid rickettsial forms. BA, Bacteria; OC, Oocyte cytoplasm. Magnification 28000x.

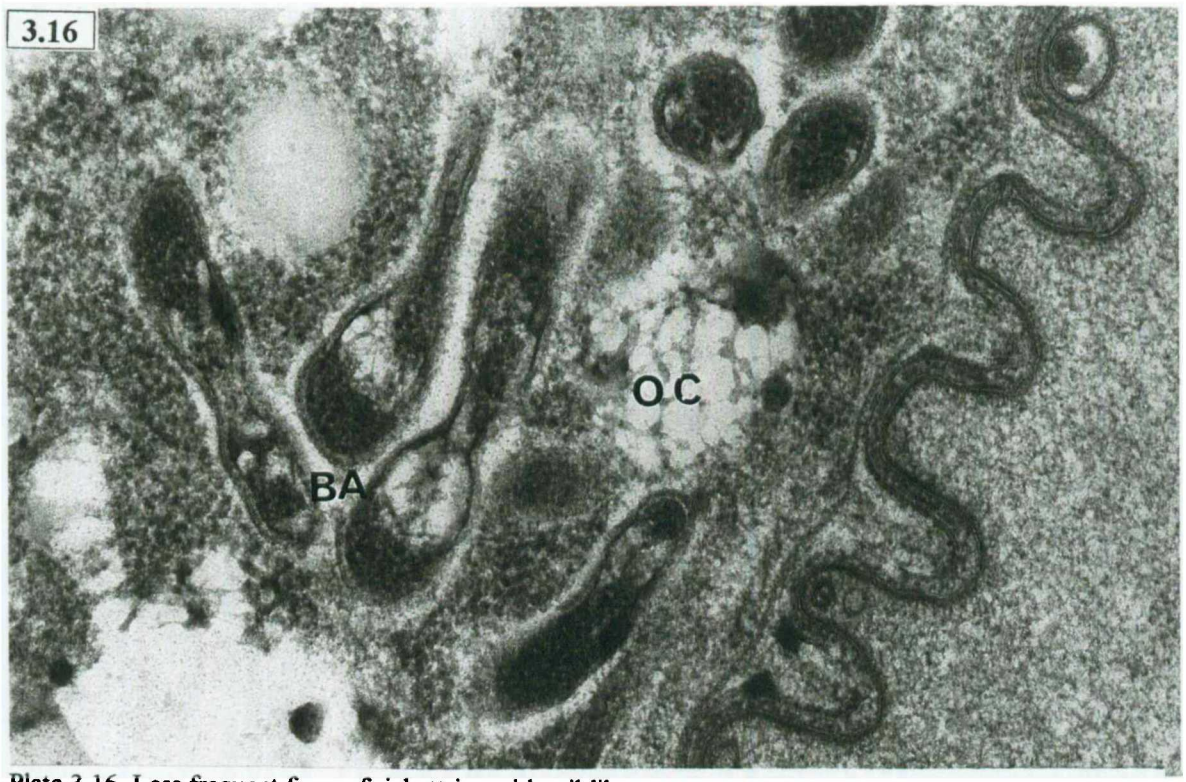


Plate 3.16. Less frequent form of rickettsiae with tail-like structure. BA, Bacteria; OC, Oocyte cytoplasm. Magnification 72000x.

There is no evidence to suggest that the different forms of the wolbachiae are related to their location within insect body. But polymorphism can be attributed to overcrowding. In late adult life of a psocid, when the rickettsiae associated with the gonads increase rapidly in number, the polymorphism becomes more apparent as more than one form is found in the crowded bacterial clusters.

3.3.5. Embryo and oocyte infection

The embryo of *L. bostrychophila* is also infected with the rickettsiae. Plate 3.19-20, shows a cross section through an embryo invaded by clusters of bacteria. The entry of the bacteria into the egg, and ultimately into the embryo varies with insect species. In *L. bostrychophila,* the primordial embryonic cells are already infected by blastoderm stage.

Since microrganisms are found in the eggs and the embryo as well as in all life stages of the insect, a tentative cycle of development is suggested. Transovarial transmissibility is confirmed and all progeny become infected via egg.

The prevalence of rickettsiae in the first instar nymphs is very low. By the time the teneral female stage is reached the densities of the microrganisms have substantially increased in the sub-epidermal region (Plate 3.18), and developing ovaries (Plate 3.3, 3.5).

Finding the *Wolbachia*-like endosymbionts in the cystocytes or in the nurse cells in early stages of ovariole development would have made tracing the cycle of the endosymbionts much easier. Of the 250 sections examined only one cut through dividing cystocytes and in this case no rickettsiae were visible. Rickettsial infection of the daughter cells is not therefore ruled out.

Soon after the oocytes enter the stage of rosette formation and the oocyte-nurse cells complex is disrupted, the oocytes becomes surrounded by follicular cells and the cytoplasm increases in volume. In this stage, quite uniformly tiny coccoids appear.

As the previtellogenic and vitellogenic phases of the egg growth proceeds, the volume occupied by the rickettsiae increases.

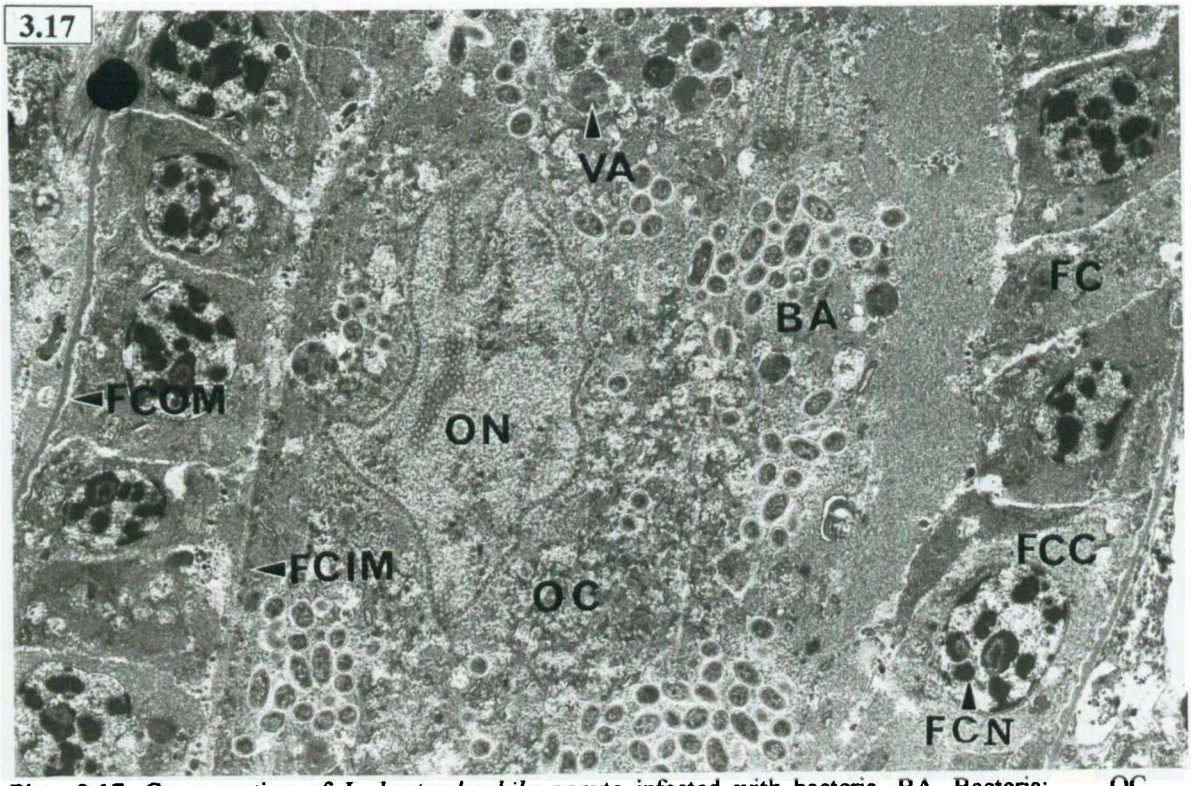


Plate 3.17. Cross section of *L. bostrychophila* oocyte infected with bacteria. BA, Bacteria; OC, Oocyte; FCOM. Follicular cell outer membrane; FCIM, Follicular cell inner membrane; ON, Oocyte nucleus; FC, Follicle cells; FCN, Follicle cell nucleus; FCC, Follicle cells cytoplasm. VA, Vacuole. Magnification 58000x.

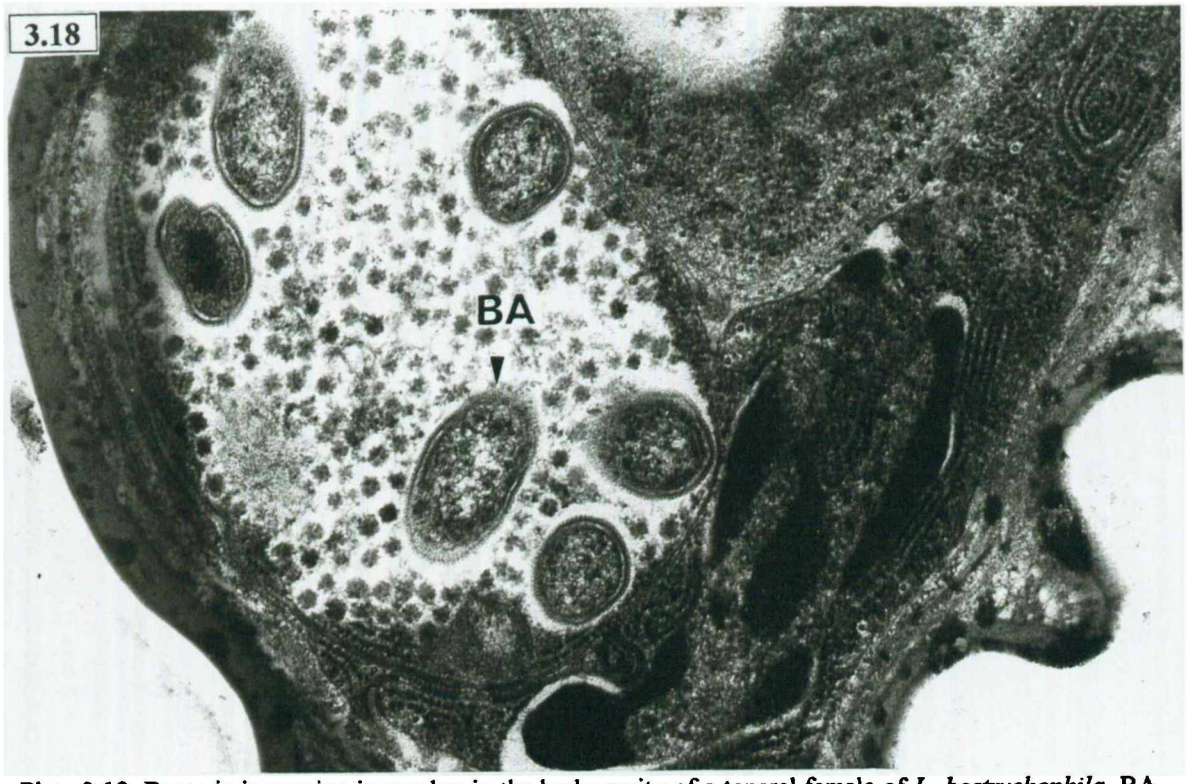


Plate 3.18. Bacteria increasing in number in the body cavity of a teneral female of *L. bostrychophila*. BA, Bacteria. Magnification 38000x.

In late stages of egg formation the density of rickettsiae increases. Vitellogenic ovary eggs were invariably infected with single or clusters of coccoids with almost identical ultrastructure and size, with diameter of 0.17μm (Plate 3.17).

In mature eggs however, the densities of rickettsiae decrease. and the bacteria become intimately associated with the developing embryo (Plate 3.19). The infection is therefore established in the eggs before they are laid (Plate 3.20). The interaction between the bacteria and embryo remain unstudied but EM micrograph observations of different individuals have shown that the cytoplasm of mature oocytes as well as laid embryos contain different densities of bacteria.

Rickettsial densities in ovaries at later stage of development can be very high, where over 80 bacterial cells were counted from a cluster of 7.14μm long and 3.2μm wide (Plate 3.9). Tissues supporting such bacterial loads show some degenerative signs. That may be due to the simple fact that the tissues could not sustain such an increase in bacterial mass and the wall between host cells seem to dissolve.

The other pathological feature shown by heavily infected cells is the amount of yolk material found in cytoplasm of the developing oocyte. The volume of the oocyte occupied by yolk material is significantly less in the heavily infected individuals (Plate 3.8).

3.3.6. Ultrastructure of bisexual psocids

Two closely related bisexual liposcelid species were also examined to see if the bacteria/psocid association is widespread. Electron microscopic investigations of the ovariole of *L. entomophila* and *L. corrodens*, show no bacterial inclusions. Plate 3.21, shows a developing ovum of *L. corrodens*, a bisexual species belonging to same sub group as *bostrychophila* in the genus *Liposcelis*. The oocyte cytoplasm is bacteria-free and full of very big yolk and lipid globules. The extent to which the size of the yolk globules are affected by the presence of this parasitic bacteria is not understood, yet there is clear difference between the oocyte content in this plate and the oocyte of *L. bostrychophila* in Plate 3.9.

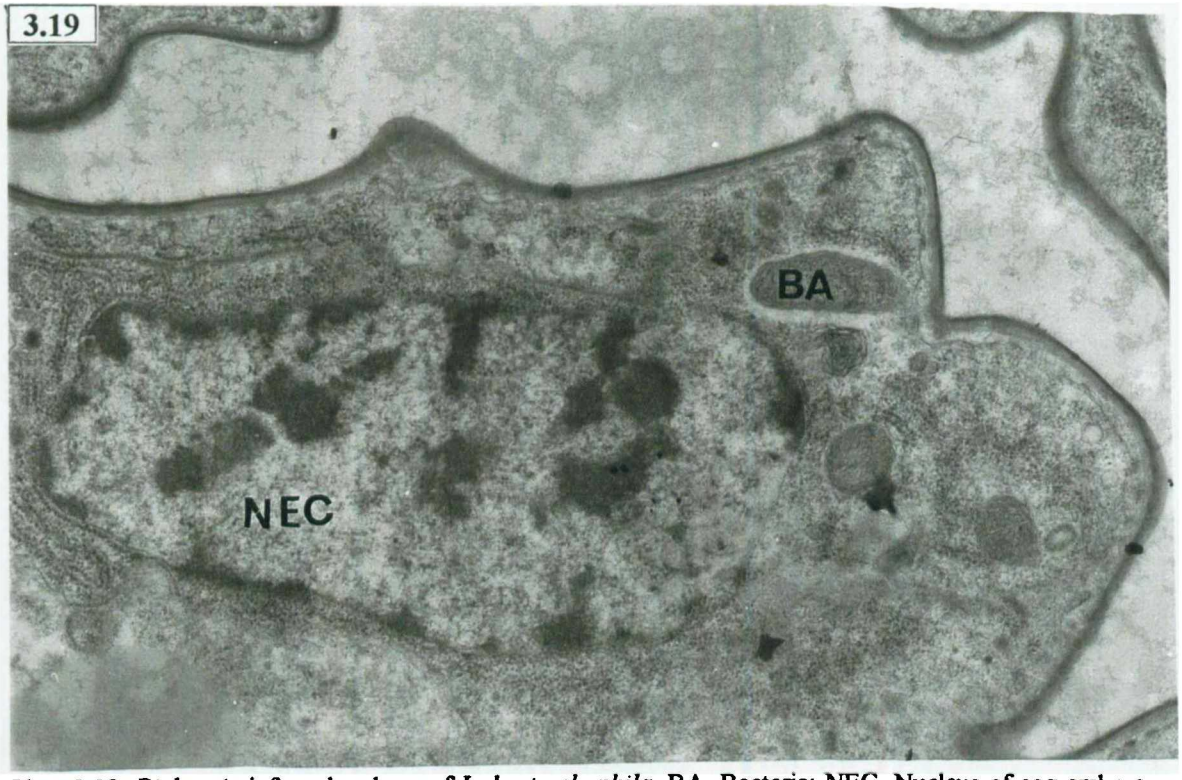


Plate 3.19. *Rickettsia* infected embryo of *L. bostrychophila*. BA, Bacteria; NEC, Nucleus of egg embryo. Magnification 20000x

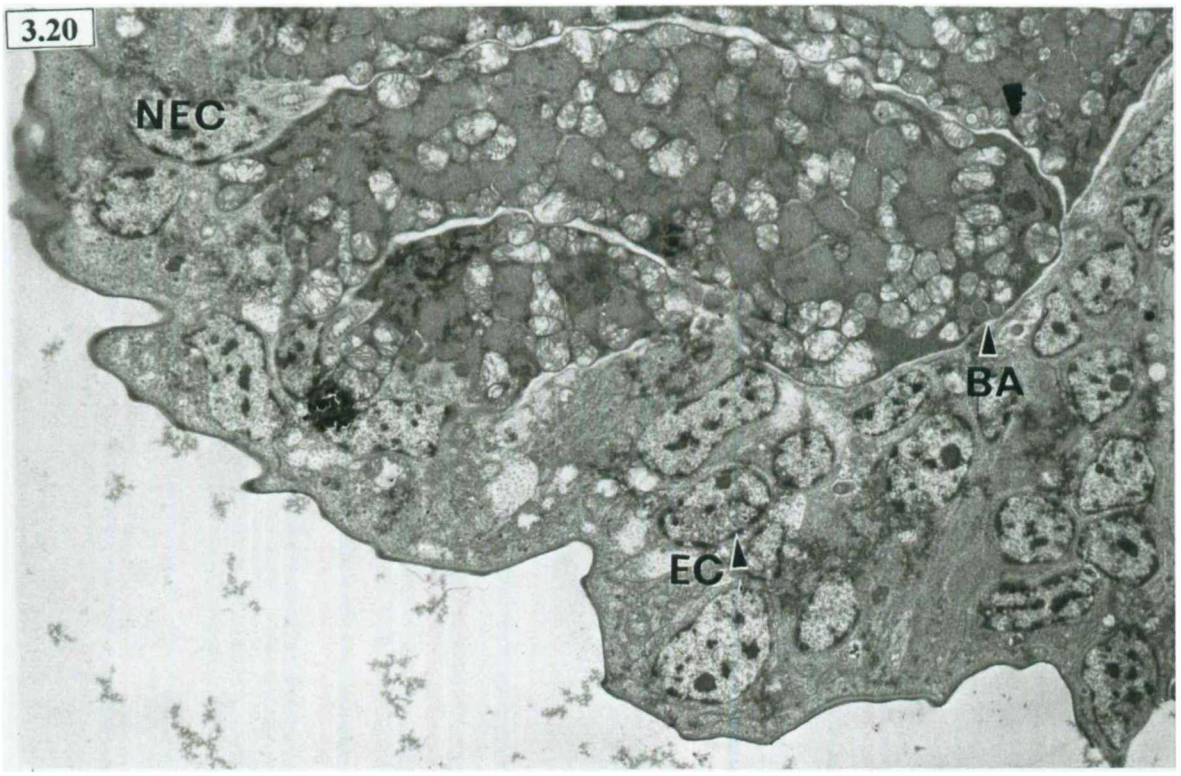


Plate 3.20. Bacterial infections in mature laid egg of *L. bostrychophila*. BA, Bacteria; EC, Embryo cells; NEC, Nucleus of egg embryo. Magnification 3800x

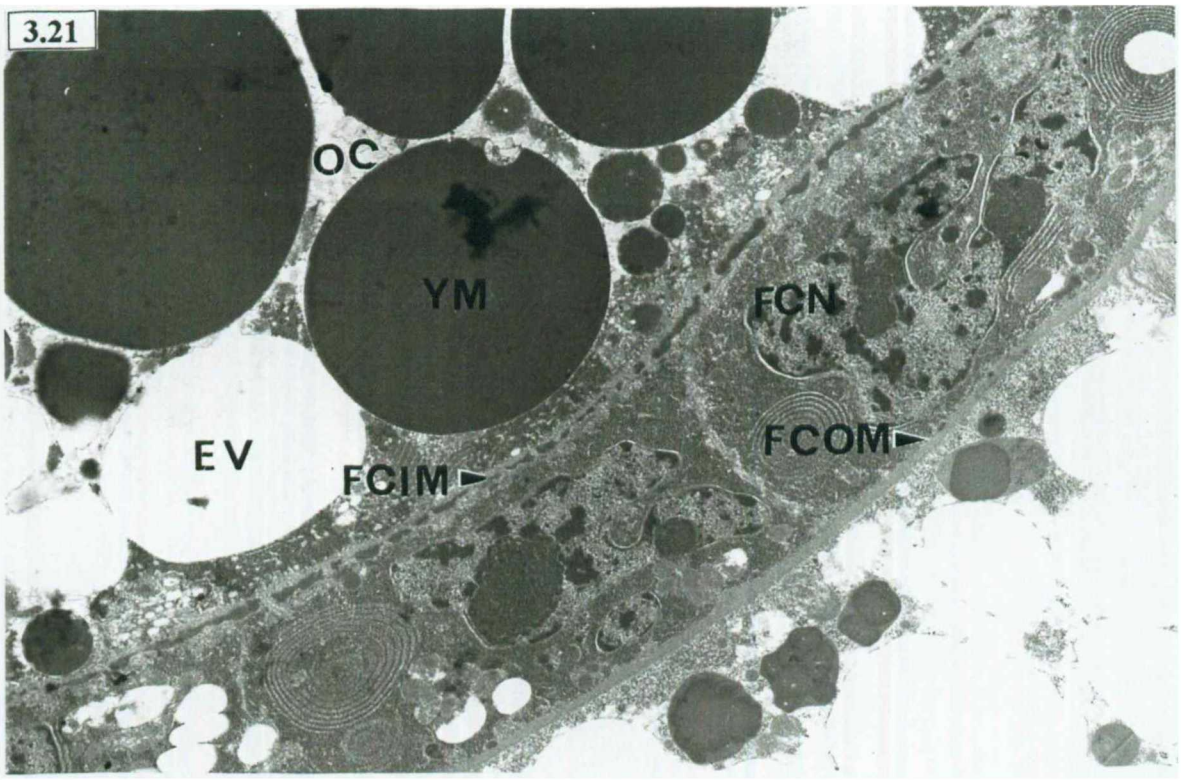


Plate 3.21. Cross section *Liposcelis corrodens* oocyte. Oocyte is healthy and shows no sign of infection. Vacuoles are comparatively larger than that of *rickettsia* laden *L. bostrychophila.* FCN, Follicle cell nucleus; FCOM, Follicle cell outer membrane; FCIM, Follicle inner cell membrane; OC, Oocyte cytoplasm; YM, Yolk material; EV, Empty vacuoles. Magnification 7200x.

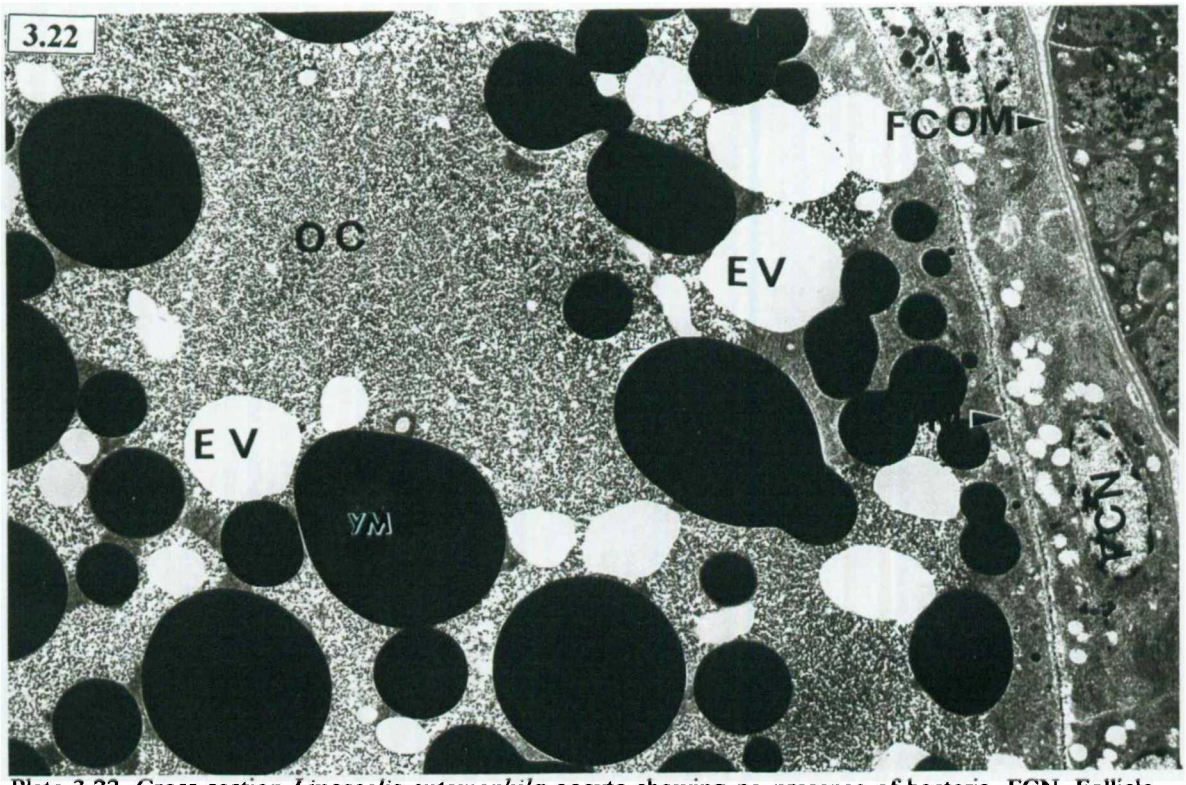


Plate 3.22. Cross section *Liposcelis entomophila* oocyte showing no presence of bacteria. FCN, Follicle cell nucleus; FCOM, Follicle cell outer membrane; FCIM, Follicle inner cell membrane; OC, Oocyte cytoplasm; YM, Yolk material; EV, Empty vacuoles. Magnification 2800x.

Plate 3.22 shows a similar cross section of the ovary of *L. entomophila,* a liposcelid belonging to a different subgroup of the genus. No bacterial cells are visible in the cytoplasm, only vacuoles filled with yolk and lipids. These observations strengthen the possibility that these transovarially transmitted *Rickettsia* induce parthenogenesis in *L. bostrychophila.*

The bisexual species were studied for comparison with the *Rickettsia*-infected parthenogens. The fine structure of the reproductive system of bisexual liposcelid species to *L. bostrychophila* was essentially similar. The ultrastructural comparison has shown that the oocytes and eggs of the uninfected bisexual species have far larger yolk and glycogen reserves than that of *L. bostrychophila* in a corresponding stage of development (Plate 3.21-22).

3.4. DISCUSSION

The ovaries and other abdominal tissues of the three liposcelid species, *L. bostrychophila, L. entomophila* and *L. corrodens,* were examined using both light and electron microscopy. Only ovarial tissues of *L. bostrychophila* are infected with *Rickettsia*-like organisms.

Light microscope results were not satisfactory due to commonly occurring granules, artefacts and other bacterium-like morphology that tended to mask the presence of the minute rickettsiae.

Culturing the rickettsial endosymbionts in a host-cell free medium would have facilitated the manipulation and undertaking further studies on these organisms but was not possible in this instance. Getting viable colonies of endosymbiotic bacteria found in the *L. bostrychophila*'s ovaries like many other fastidious prokaryotes proved to be impossible with the techniques used.

Bacteria infecting the ovaries of the psocid *L. bostrychophila* were found to be highly polymorphic and vary in shape from spherical to elongate.

Polymorphism is recognised as one of the main features of the rickettsial endosymbionts with different researchers using various methods. Nevertheless it is possible that the variations in structure observed might be affected by the sectioning process. Spherical profiles represent cross-sections of both elongate forms and coccoids. Most of the longer microrganisms appeared to be dividing. Forms which may be designated as classical or typical rickettsiae, i.e., tiny coccoids grading into short, straight rods of approximately equal diameter occurring in clusters are always found. Enlarged coccoids and *Wolbachia*-like organisms (WLO) with tail-like structure have also been encountered less frequently. The shape and size of WLO are not thought to depend on the site of the bacteria. More than one form can be encountered in one location.

It is difficult to assign any form as the standard. Forms, which may be designated as classical or typical rickettsiae, i.e., tiny coccoids grading into short, straight rods of the same diameter with characteristic ultrastructure, occur singly or in clusters and are always present. Bacteria were not found to be randomly dispersed in the ovary, but concentrated in the cytoplasmic matrix of the oocyte and organised in a mosaic fashion.

Similar ultrastructural investigations in mosquitoes *Aedes* have shown rickettsiae in the oocyte nurse cells and cystocytes (Wright and Barr, 1980a; Subbarao, 1982). In *Aedes scutellaris, Wolbachia*-infected cystocytes have intercellular bridges, wide enough to allow the passage of microrganisms from one cell to another (Wright and Barr, 1980a). On the one occasion that *L. bostrychophila* cystocytes are sectioned, no rickettsiae were found. In the *Culex pipiens* complex, *Wolbachia*-like organisms were found in oocytes and nurse cells but not in follicular cells (Subbarao, 1982). Binnington and Hoffmann (1989) in their study of the fruitfly *Drosophila simulans* found WLO only in the oocytes. Hertig (1936) reported that Wolbachiae were seen extracellularly below the muscle and nearby areas of the mosquitoes, as well as in the gonads and malpighian tubules. Much later examinations confirmed the presence of rickettsiae in all these tissues but failed to find any extracellular presence (Wright and Barr, 1980b). It therefore appears that the WLO occupy a number of tissues in insects and that the sites of infection vary among species. As part of this study, 31 populations of *L. bostrychophila,* collected from different part of the country, were examined.

The invariable occurrences of this rickettsial endosymbionts have been confirmed in all populations examined. Wolbachiae were found specifically in the developing oocyte and in the subcutaneous tissues of *L. bostrychophila*. The nurse cells, follicle cells, enveloping membranes and other epithelium and muscular tissues associated with ovarioles and the gut tissues were not infected with the bacteria.

CHAPTER FOUR

EXPERIMENTAL ATTEMPTS TO ELIMINATE RICKETTSIAL ENDOSYMBIONTS FROM *L. BOSTRYCHOPHILA*

CHAPTER FOUR

4.0. EXPERIMENTAL ATTEMPTS TO ELIMINATE RICKETTSIAL ENDOSYMBIONTS FROM *L. BOSTRYCHOPHILA*

4.1. INTRODUCTION

Whilst the bisexual liposcelids show no evidence of harbouring rickettsiae, the bacteria have been seen in every specimen of *L. bostrychophila* examined. On the basis of the nutritionally balanced diet, the lack of membrane surrounding the bacteria and the site of infection, it appears unlikely that the endosymbionts are mycetomal in *L. bostrychophila.* The evidence presented here has shown that the endosymbionts in *L. bostrychophila* are intraoocytic *Wolbachia*-like infections belonging in the family *Rickettsiae.*

The usual technique for demonstrating the significance of an endosymbiotic relationship consists of eliminating the microrganisms participating in the symbiosis and studying the effects produced in the hosts by their elimination. Removal of nutritionally important endosymbionts in the cockroach *Blatella germanica* produced offspring with reduced vigour (Brooks and Kringen, 1972). Heat and antibiotic treatments are both established methods of producing, *Rickettsia*-free individuals (Steinhaus, 1949; Wade, 1984; Hoffman, 1988; O'Neill and Karr, 1990). Experimental treatments designed to produce *Rickettsia*-free psocids, termed aposymbiotic, were carried out during the course of this study using these same heat and antibiotic methods.

The results of those treatments are presented in this chapter. Egg output data and electron microscopic examinations of serially sectioned treated and untreated eggs and adults are compared.

The bisexual liposcelid species, naturally lacking the *Rickettsia*-like bacteria, were used as controls to detect any adverse effects of antibiotic and heat treatment on psocid reproduction physiology and survival.

Norris (1972) reported the case of transovarially transmitted endosymbiotic rickettsiae in the asexual ambrosia beetle *Xyleborus ferrugineus.* In this insect the presence of an adequate population of the bacteria are needed for embryonic development in the egg.

This author suggested that the activation of mature oocyte, a function of normal sperm penetration of the oocyte, is assumed by the bacterial symbionts.

Rickettsiae are known to be a heat sensitive group of organisms and cannot survive temperatures above 33°C (Steinhaus, 1949). Experiments involving the thermal treatment of endosymbionts of *Sitophilus*, by holding the infected insects at 32°C or more, produced aposymbiotic females (Musgrave and Miller, 1951; Werren, 1997). Others authors reported failure (Dang-Gabrani, 1970), creating conflicting results on the efficiency of heat treatment. Failures have been reported in *Oryzeaphilus surinamensis* (Kolya and Pant, 1962), *Blattella germanica* (Brooks and Richards, 1955) and *Sitophilus* (Musgrave *et.al.*, 1963). The efficiency of using elevated temperatures as a means of eliminating bacterial inclusions in insect tissue is not reliable as the thermal sensitivities of symbionts are poorly understood (Pant and Dang, 1972).

Wide ranging antibiotics with different modes of action have been used to produce aposymbiotic insects. The most widely used ones include tetracycline, streptomycin, rifampicin, erythromycin, potassium penicillin, methyl bromide, aureomycin (Norris, 1972; Pant and Dang, 1972; Stouthamer *et.al.*, 1993). All these were reported to have eliminated bacterial endosymbionts with various degrees of success and impact on the insect host. In this chapter, results of the use of two of them, namely tetracycline and rifampicin are presented. The antibiotics tetracycline together with erythromycin and spectinomycin inhibit the prokaryotic and mitochondrial protein synthesis by binding to the ribosome (Wade, 1984). Rifampicin on the other hand is a more potent inhibitor of DNA-dependent RNA polymerase of bacteria.

The physical or chemical means chosen for administering the antibiotics to eliminate endosymbionts depends on the nature of the symbiosis to be investigated (Nogge and Gerresheim, 1982).

Insects which spend some stage in their life cycle in water, like mosquitoes, are best suited for antibiotic treatment. *Wolbachia* infected mosquito larvae raised in water containing tetracycline are cured of the microrganisms (Wade, 1984).

The oral administration of antibiotics with food was the only practical dosing route, given the minute size of liposcelids. Other methods of introducing antibiotics to larger insects

include injecting antibiotic solutions (Richardson *et. al.*, 1987). Since it was impossible to culture the *Wolbachia*-like organisms in a cell free medium, they could not be tested *in vitro*. Administering antibiotics to intracellular bacteria is the routine method of producing a partial or complete aposymbiotic condition. Many successful chemotherapeutical eliminations of endosymbionts have been reported (e.g. Norris, 1972; Wade 1984; Stouthamer *et.al.*, 1993; Wilkinson and Douglas, 1996; Eichler and Schuh. 1998; Caillaud and Rahbe, 1999) as well as failures (eg. Brooks and Richard, 1955).

In some insects the endosymbionts are surrounded by host membrane and the possibility of protection by the insect has been suggested (Pant and Dang, 1972). In these cases the microrganisms are resistant to antibiotics. Host defence might be the only explanation in the case of the symbionts of the stored-product beetle *Sitophilus oryzae*. In this species bacterial endosymbionts closely resembling *Bacillus* sp. were found to be resistant to the action of potent antibiotics, whilst similar species of bacteria associated with higher animals, are susceptible (Dang-Gabrani, 1970; Dang, 1971).

An important discovery after using antibiotics was achieved when Stouthamer *et.al.*, (1993) discovered that strains of parthenogenetic *Trichogramma* wasps revert to sexual reproduction as result of the successful elimination of rickettsial endosymbionts from the wasp gonads.

4.2. MATERIAL AND METHODS

4.2.1. Experiments using antibiotics and heat to eliminate endosymbionts

Newly maturing female psocids, that is when the colourless teneral stage begins to change to brown (12-24 hours after the final moult), were removed from the culture with a fine brush. Individuals were placed singly in glass vials containing a piece of filter paper which had food mixed with antibiotic of known concentration glued on one side. The standard diet consisted of yeast and flour in ratio of 2:1. The glass vials were covered with fine mesh net to prevent the psocids escaping.

Antibiotic agents used were tetracycline-HCl (BDH) in crystal form and powdered rifampicin (Sigma). These are two of the most widely used antibiotics for this purpose.

Concentrations of antibiotics, shown to eliminate similar insect endosymbiotic bacteria (Nogge and Gerresheim, 1982; Pant and Dang, 1972; Brooks, 1972) were prepared. Final concentrations of tetracycline and rifampicin were calculated by mixing 10g of the standard diet with 0.1g of either tetracycline or rifampicin. Then 3g of this food-antibiotic mixture was further diluted into 10g and 100g of standard diet to make final concentrations of 1%, 0.3% and 0.03% in the standard diet. A combined treatment of both tetracycline and rifampicin was prepared in similar fashion to make up a 1% total mix of antibiotics in the standard diet. Initially the food and antibiotic were mixed dry but this produced patchy results. Subsequently the food was moistened with sterile distilled water and then suspended with the antibiotic in the powder form to create a thoroughly homogeneous slurry which was then air dried and ground to a powder. This uniform preparation was fed to the insects and gave better results in antibiotic uptake.

Twenty psocids were used for each antibiotic treatment experiment. Glass vials, each containing a single psocid female, were placed into a plastic container with saturated sodium chloride to regulate the relative humidity.

A similar number of replicates at the same temperature and relative humidity were used as controls, but fed standard diet alone.

4.2.2. Series of experiments in antibiotic treatments

The following series of experiments were carried out:

Series A: Antibiotic treated nymphs

As soon as nymphs emerged from freshly laid eggs, 20 were transferred to individual containers containing filter paper of which one side had a mixture of food and antibiotic (at 1%, 0.3% and 0.03% concentration) glued to it. These nymphs were reared on this mixture until they become adults. These experiments were used to monitor the effect of antibiotics on juvenile development and mortality.

Samples of nymphs at different developmental stages were taken out to find out if the infection of the rickettsiae was still present. Once they had become adults, they were

monitored for egg production and kept for a further 6-7 weeks on the antibiotic laced diet. Eggs were counted on a weekly basis.

Some of these adults and eggs were killed and examined for the presence of the bacteria.

Series B: Antibiotic treated adults

Newly moulted females were isolated from cultures and given the antibiotic laced diet as in series A. These individuals had been reared on standard food as juveniles, and were assumed to be all infected with rickettsiae. Adult egg output, mortality and egg hatchability were monitored. Eggs laid by these females were sectioned and examined using TEM to detect intracellular bacteria. The effect of antibiotics on psocid fecundity was also observed.

The experiments in series A & B were paralleled with controls using *L. bostrychophila* of similar age and population. Control individuals were fed on the standard diet, held on same temperature, % rh. regime and monitored in a similar fashion.

Series C: Attempts at establishing *Rickettsia* free population

Small populations were kept in glass jars containing food mixed with tetracycline and rifampicin for several months. The antibiotic concentrations were the same as those in the previous series of experiments. These experiments were designed to determine how long it might take before the *Wolbachia*-like bacteria could be successfully removed from the psocid populations, and whether males might be produced.

Eggs from these females were harvested from these populations to determine juvenile development and hatchability. For the purpose of analysis all nymphs emerging within the same period were treated as a cohort.

Individual eggs were checked for the presence of bacteria (where and how many) the initiation of primary oocyte(s) and whether the nucleus of such oocytes showed any cell division, from sections viewed by transmission EM. Electron micrographs of follicles and oocytes of treated and untreated liposcelids were also examined for any ultrastructural differences.

These three series were repeated using the bisexual species *L. corrodens and L.*

entomophila as controls for the effect of antibiotic treatment on reproduction and survival in non-rickettsia bearing liposcelids.

4.3. RESULTS

In general the elimination experiments provided varying degrees of success. Individuals from three different populations were each subjected to three tetracycline concentrations and two high temperature regimes (37°C treated and control). Similar experiments using rifampicin were more successful. The data from juveniles reared on antibiotic-laced diet, mortalities and adult egg production are summarised in appendix tables A4.14-16.

Analysis of variance on these data showed significant differences in egg production between tetracycline treated individuals and controls (Table 4.1).

Due To	Sum of Squares	DoF	Mean Square	F-Stat	Signif
Main Effects	2508.056	15	167.204	29.687	0.0000
time	1877.294	9	208.588	37.035	0.0000
concentrat	220.456	5	44.091	7.828	0.0000
population	108.643	1	108.643	19.290	0.0000
2 Way Interactions	1556.502	44	35.375	6.281	0.0000
time × concentrat	1167.975	40	29.199	5.184	0.0000
time × population	214.929	9	23.881	4.240	0.0000
concentrat × population	14.449	1	14.449	2.565	0.1096
3 Way Interactions	0.000	0			
time × concentrat × population	44.246	9	4.916	0.873	0.5491
Explained	4108.804	14	293.486	52.109	0.0000
Error	4922.546	874	5.632		
Total	9031.350	888	10.170		

Table 4.1 : ANOVA table for the egg production of different *L. bostrychophila* treated with different concentration of tetracycline.

In this table the 3 way analysis of variance shows that time, concentration of tetracycline and strain of *L. bostrychophila* had a significant impact on the egg production. The interaction between time/concentration, time/population and concentration/population was also significant. Nevertheless the interaction between all three (time/concentration/population) was not significant.

Cohort egg production of 0.03% tetracycline treated individuals and controls at 25°C totalled 217 in 0.03% tetracycline treated and 309 in *L. bostrychophila* fed standard diet in 10 weeks (Table A4.1-4.4).

Fig. 4.1, shows the egg production of *L. bostrychophila,* of population 01, at 25°C. The tetracycline (0.03%) treated psocids produced a consistently lower number of eggs then the controls. Population 05, kept in similar conditions, gave the same pattern(Fig 4.2).

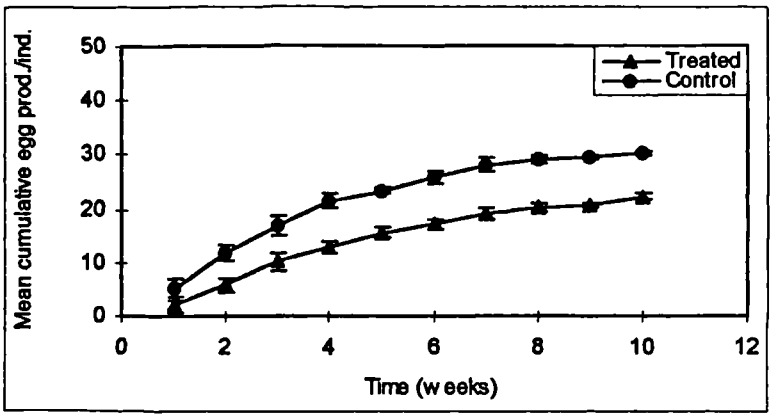


Fig.4.1. Egg production (mean ±2SE) of *L .bostrychophila* (population 01) treated with 0.03% tetracycline at 25°C and controls.

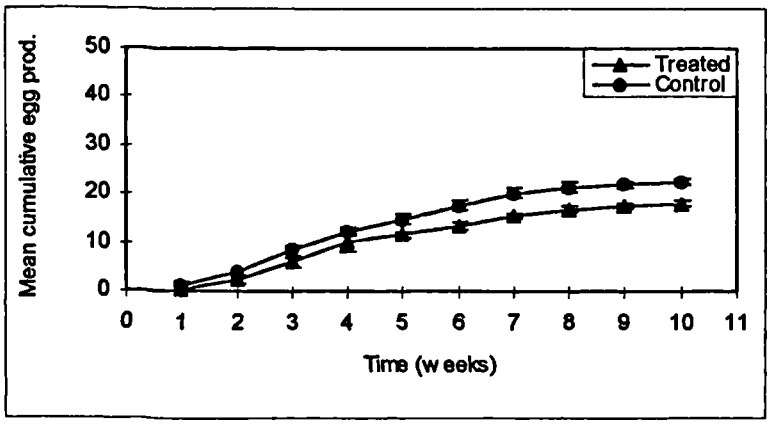


Fig.4.2. Egg production (mean ±2SE) of *L .bostrychophila* (population 05) treated with 0.03% tetracycline at 25°C and controls.

Another set of experiments was carried out using two higher concentration of tetracycline in psocid diet (0.3% and 1%,) at a high temperature of 37°C. The high temperature and high concentration of tetracycline (1%) treatment were combined to achieve enhanced

'curing'. Both techniques are established treatment tools for intercellular *Rickettsiae*. The results of these combined heat and antibiotic treatments are shown in Tables A4.5-8.

Egg production of *L. bostrychophila* is very rapid at 37°C regardless of the level of tetracycline administered. The lowest egg production was obtained in the highest dose of antibiotic at 1% and the difference is statistically significant. The distribution of egg production in ten weeks in tetracycline administered individuals is nevertheless significantly different from controls. Egg production by antibiotic treated females was delayed and peaked in the 3^{rd}-4^{th} weeks before dropping sharply (Table A4.1-4.4).

In control individuals, egg production starts in the first week reaching peak output as early as the second week. This high output is maintained up to fourth to fifth week, before it declines slowly according to the degree of temperature and the population. In the heat-treated *L. bostrychophila* (no antibiotics) incubated at 37°C, the individual egg output was highest in the first week (Table A4.5). From then on the number of eggs per individual per week declines gradually until the fourth week. Similar patterns were not seen when psocids were fed with a tetracycline-laced diet. As seen in figure 4.3, the accumulated egg production from heat and standard diet fed females rises and slowly levels off from the sixth week. The tetracycline treated females level off from the fourth week. The slope of the cumulative egg production in 1% and 0.3% tetracycline is almost identical.

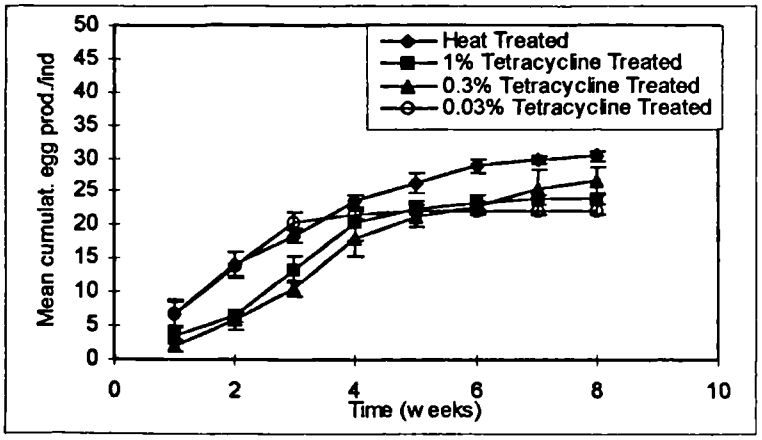


Fig.4.3. Egg production (mean ±2SE) of *L .bostrychophila* (population 09) treated with heat at 37°C and an increasing dose of tetracycline (0.03, 0.3 and 1%).

Individuals treated with 0.03% tetracycline and controls produce equal number of eggs, indicating that such a low concentration of tetracycline has no impact on egg output.

Similarly there is little difference in the mortality rate between 0.03% tetracycline treated and controls at 25°C and 37°C in all three *L. bostrychophila* populations. These are shown on Fig. 4.4-6. The rate of mortality stays the same at 60% after 10 weeks when experiment were terminated (N=20) (Fig 4.4-5). The concentration of tetracycline used did not have any effect on the level of adult mortality. In all three concentrations of tetracycline together with controls, the rate of mortality stayed between 60-65 % at lower temperature range of 25°C. Mortality rate increased in the experiments with higher temperature and tetracycline concentrations.

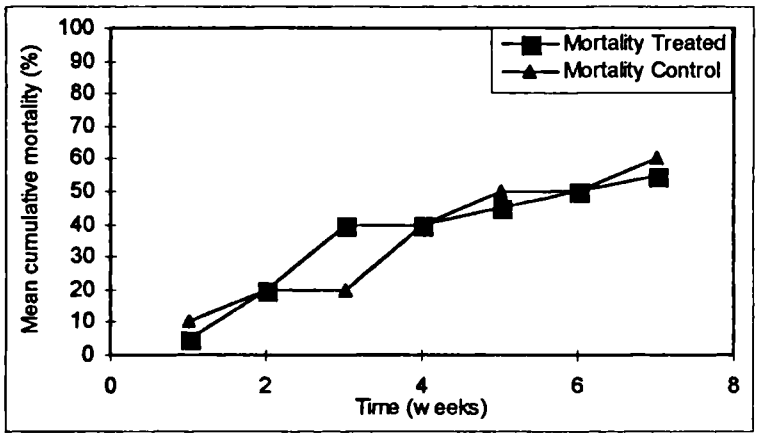


Fig. 4.4 Cumulative mortality of *L. bostrychophila* (population 05) treated with 0.03% tetracycline and controls.

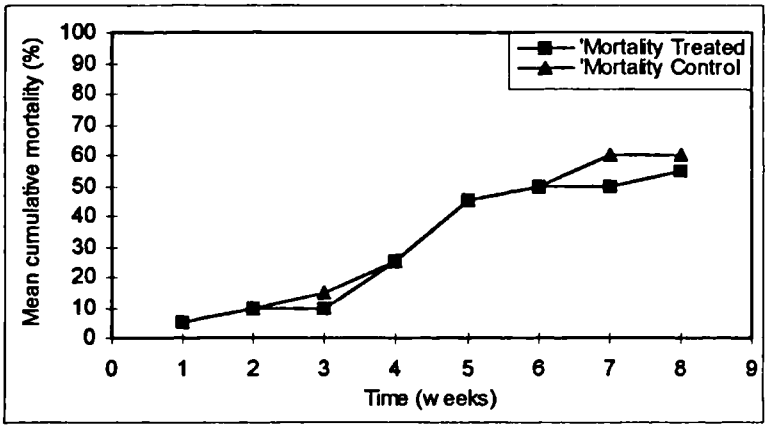


Fig. 4.5. Cumulative mortality of *L. bostrychophila* (population 01) treated with 0.03% tetracycline and controls.

At 37°C the highest mortality occurred at 1 % concentration of tetracycline (Fig. 4.6) where 80% of females died after 10 weeks. Lowest mortality was achieved at 0.03% tetracycline at 37°C, followed by 0.3%.

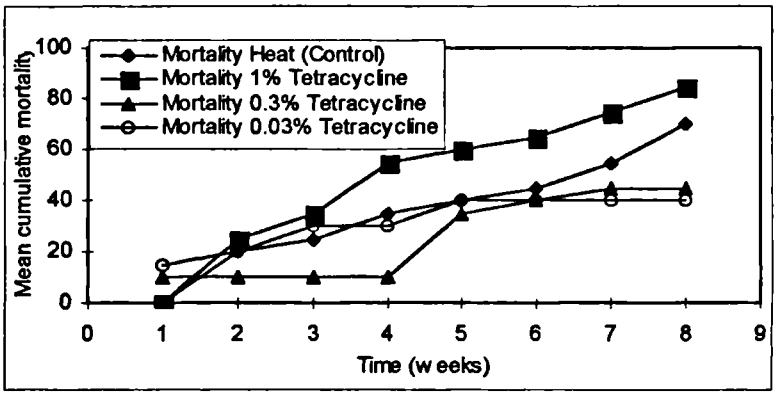


Fig. 4.6. Cumulative mortality of *L. bostrychophila* (population 09) treated with heat at 37C (control) an increasing concentration of tetracycline (0.03, 0,3, 1% tetracycline).

4.3.1. Histology of heat and tetracycline treated oocytes.

Heat treatment at 37°C was not enough to render infected *L. bostrychophila* aposymbiotic. But the oocytes of females that received prolonged heat treatment showed a sharp decrease in bacterial density. Plate 4.1 shows a cross section of mature oocyte kept at 37°C for more than 6 weeks. The insect was fixed and sectioned in the seventh week of egg production. The rate of egg output of the sectioned individual, as well as most others at 37°C was high in the first three weeks, and then sharply reduced in the fourth week. The egg production was nil by the week 6.

Plate 4.1 shows fully developed oocyte, with 0.28μm thick basement membrane, surrounded by active secreting follicle cells containing chromatin material fully extended. The cytoplasm of such an oocyte lacks the dense aggregations of bacterial cells so typical of *L. bostrychophila* ovaries. The evidence of the endosymbiont's destruction that have occurred can be easily visualised in the highly electron-translucent area occupied by the microrganisms. The remaining bacteria are still visible in the electron-translucent area. Heat also served as means of gradually decontaminating the insect tissues in subsequent generations. The nymphs grown in this critical temperature had enough bacteria for reproduction but the load remained low.

Plate 4.1. Cross section of a mature, heat treated *L. bostrychophila* oocyte. The oocyte has been made partially aposymbiotic by high temperature (37°C). FC, follicle cells; FCN, follicle cell nucleus; BA, bacteria; EV, empty vesicles; YM, Yolk material; OC, oocyte cytoplasm; BM, basement membrane. Magnification 10800x.

At 37°C the laboratory strains produces a high initial burst of eggs but the production rapidly drops back. This corresponds to the reduction of the rickettsiae in the eggs.

High temperature enhanced the activity of the antibiotics. High metabolic rate will lead to an increased rate of feeding and therefore higher uptake of the antibiotic. Heat and antibiotic treated oocytes did not show any noticeable ultrastructural changes, apart from the loss of the rickettsiae.

Electron microscopy sections of oocytes of tetracycline treated and untreated controls (that received neither antibiotic nor elevated temperatures) were examined and compared. Occasionally the treated eggs showed an increased vacuolisation and a patchiness of the cytoplasm. Tetracycline treatments alone failed to produce complete aposymbiosis. Bacteria were still present in the oocyte of 1% tetracycline treated individuals albeit in low densities (Plate 4.5). The ultrastructures of these bacteria are markedly different from those found in the untreated ones. Plate 4. 2, shows bacterial cells in the treated oocytes displaying various degrees of degeneration. The nuclear material of the bacteria is completely dissolved in most cells (Plate 4.3). In many instances the cell wall loses its rounded coccoid structure but remains largely intact (Plate 4.4). Oocytes treated either with heat or antibiotics show an immediate reappearance of large globules of yolky material in the oocyte cytoplasm (Plate 4.1-4.4). The area of emergence of the yolk material is of interest; it appears to occur in areas where the microrganisms were eliminated. The oocyte of *L. bostrychophila* is built in a highly mosaic fashion and bacteria clusters are seen only in the cytoplasm. The emergence of the yolk globules appear to be directly related to the disappearance of the bacteria, there may be a relationship between synthesis of yolk components and bacterial metabolism.

4.3.2. Rifampicin treatment results

Rifampicin proved to be a more potent antibiotic on *L. bostrychophila* symbionts than tetracycline. Rifampicin successfully produced a completely aposymbiotic condition (Plate 4.6). The results of the egg production of rifampicin treated psocids are shown in Tables A4.9-13.

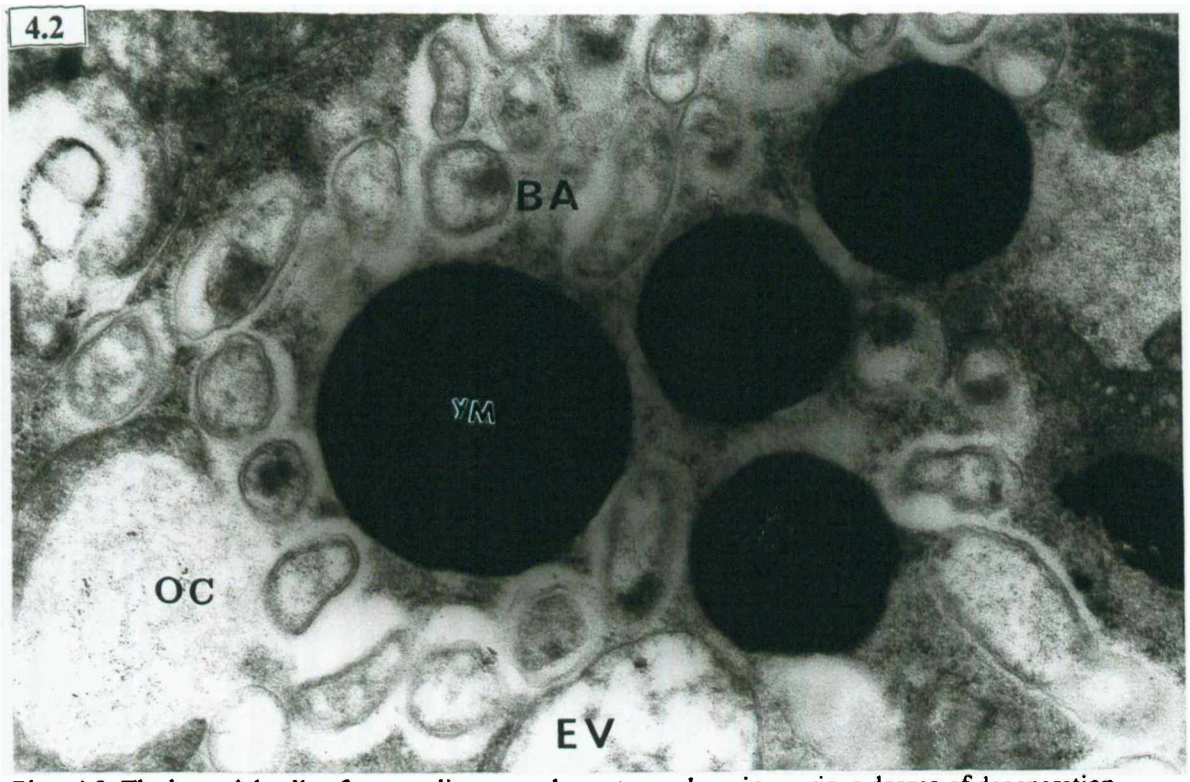


Plate 4.2. The bacterial cells of tetracycline treated oocytes undergoing various degree of degeneration. BA, bacteria; EV, empty vesicles; YM, Yolk material; OC, oocyte cytoplasm. Magnification 28000x.

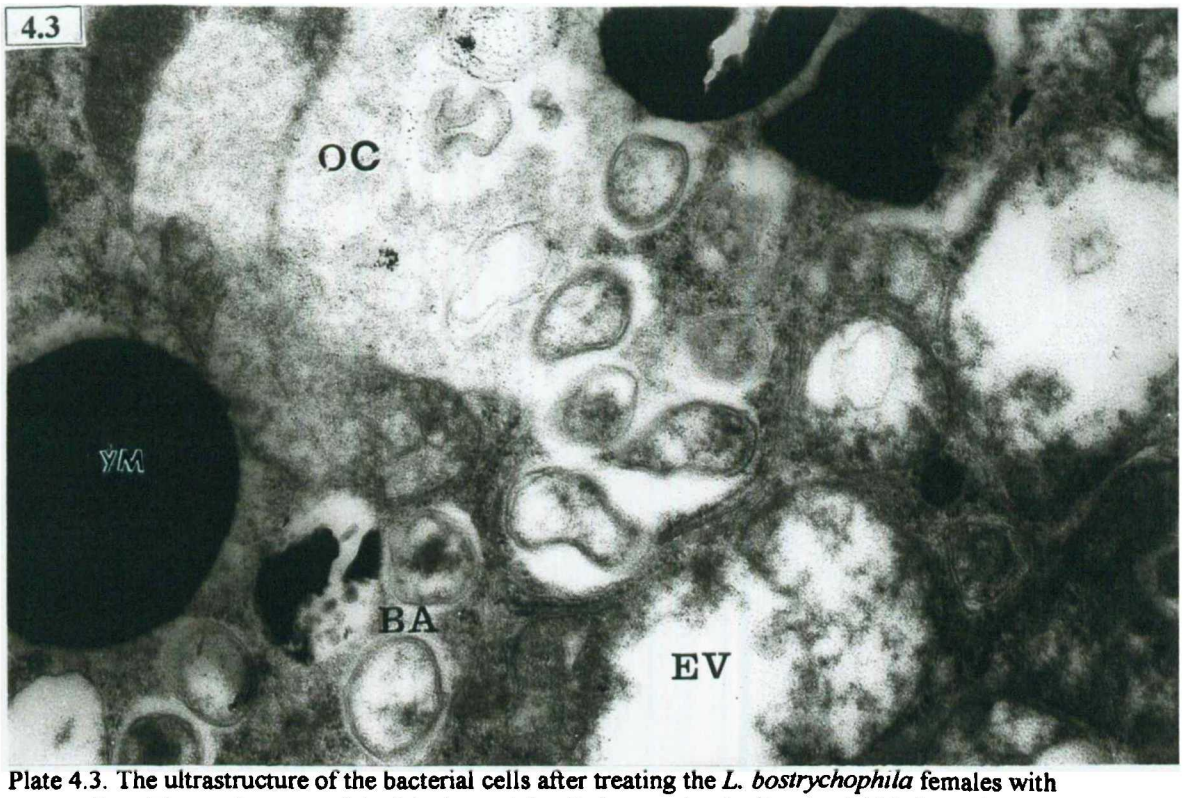


Plate 4.3. The ultrastructure of the bacterial cells after treating the *L. bostrychophila* females with tetracycline. The nuclear material of the bacterial cells is completely dissolved. BA, bacteria; EV, empty vesicles; YM, Yolk material; OC, oocyte cytoplasm. Magnification 28000x.

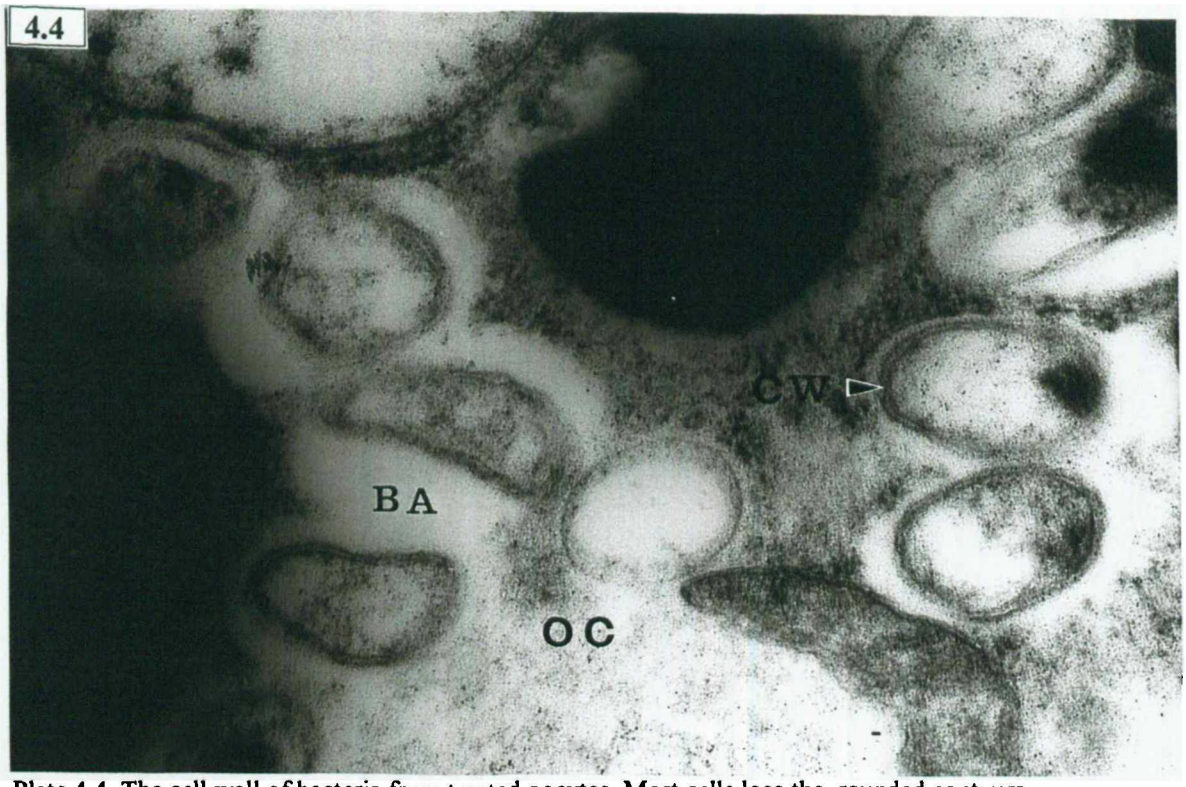


Plate 4.4. The cell wall of bacteria from treated oocytes. Most cells lose the rounded contours characteristic for the coccoid rickettsiae but the cell wall remains largely intact. BA, bacteria; CW, cell wall; , OC, oocyte cytoplasm. Magnification 58000x.

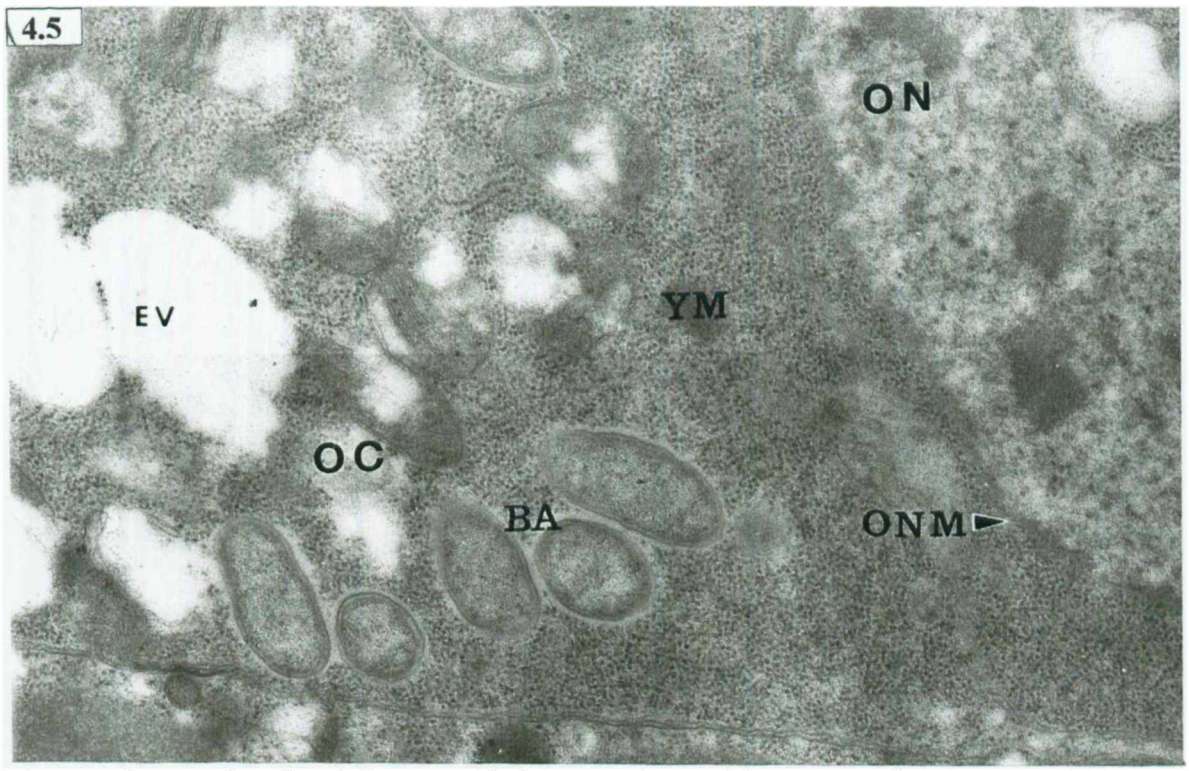


Plate 4.5. Cross section of partially aposymbiotic oocyte after receiving a tetracycline treatment. The bacteria cells although seriously reduced in number are still visible. BA, bacteria; YM, Yolk material; OC, oocyte cytoplasm; ON, oocyte nucleus; ONM, oocyte nuclear membrane. Magnification 28000x.

Seven weeks of egg production of *L. bostrychophila* treated with rifampicin or combined rifampicin-tetracycline was recorded and compared with controls fed standard diet without antibiotics. Analysis of variance of these data is summarised in table 4.18. This table shows that both treatment and time were highly significant and fundamentally affected the egg production.

Due To	Sum of Squares	DoF	Mean Square	F-Stat	Signif
Main Effects	4207.559	9	467.507	59.022	0.0000
time	558.459	6	93.076	11.751	0.0000
treatment	3707.293	3	1235.764	156.013	0.0000
2 Way Interactions	719.721	18	39.984	5.048	0.0000
Time × treatment	719.721	18	39.984	5.048	0.0000
Explained	4927.279	27	182.492	23.039	0.0000
Error	2407.950	304	7.921		
Total	7335.229	331	22.161		

Table 4.18. ANOVA table for the egg production of different *L. bostrychophila* treated with rifampicin.

Rifampicin treated females showed a dramatic reduction of egg output (Table A4.9 and A4.11). Psocids did not produce any eggs in the first week and almost none in the second week. The highest mean number of eggs per individual of 1.5 eggs (*N=20*), was achieved in the third week and egg production ceased in the fifth week. 45 % of the treated females did not lay any eggs. A further 25% laid only 1-2 eggs in the 7 weeks before the experiments were terminated. The number of eggs laid by the rifampicin treated females totalled 56 with half of the individuals dying after the seventh week. The egg production of the controls without antibiotics at 30°C was about 10 times higher. Egg laying started in the first week and peaked early in the second week where the mean number of eggs per individual reached seven. Mean production per insect slows down to 5 in the third week before it shows a second peak in the fourth week where almost 10 eggs were laid per female.

Unlike the antibiotic treated individuals, the controls continued laying eggs until the experiments were terminated in the seventh week.

Fig. 4.7, shows the total egg production of rifampicin treated and controls respectively. The combined treatment of rifampicin and tetracycline did not achieve a more rapid method of producing bacteria-free psocids than rifampicin alone. The egg production of the combined treatment females was similar to those treated with rifampicin and not

those treated with tetracycline alone.

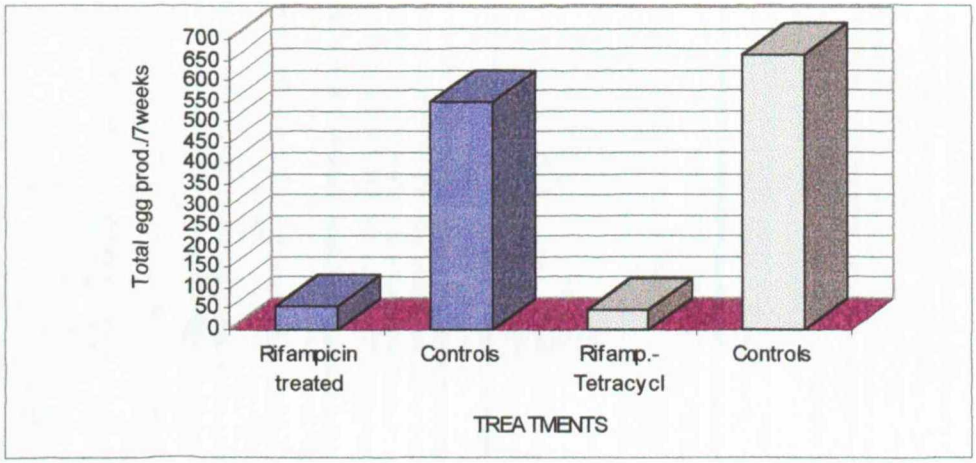


Fig. 4.7. Total egg production of *L. bostrychophila* treated with 1% rifampicin, rifampicin-tetracycline and their controls.

This coupled with, the production of aposymbiotic females in rifampicin treated individuals indicated that, rifampicin was more potent in eliminating the *L. bostrychophila* symbionts. All 56 eggs laid by rifampicin treated females in this experiments were kept and monitored. The eggs were removed from vials and placed in new containers where emerging nymphs were fed on the same antibiotic-laden diet given to their mothers. Egg hatchability was low measuring 39.2% (*N=56*). About 32% (*N–22*) of the newly emerged nymphs survived. Only two individuals survived in this instance to reach adulthood. The two females were killed and examined with TEM. No bacteria were visible in their ovaries. The combined treatment reduced the egg production by 14 fold with 50 % of treated females (*N–20*) failing to initiate egg laying, while a further 10 % only laid a single egg throughout the seven weeks of observations. The mean number of eggs per individual per week was, as a result, very low and reached only one egg in the second week and in all subsequent weeks it was always less than that. In contrast, the controls followed the typical *L. bostrychophila* egg-laying pattern. Egg production of the controls peaked in the second-third week and gradually slowed down from almost two eggs per day/female in the third week to approximately 4 per week in the seventh week (Table A4.13). TEM analysis of the ovaries of females treated with the combined rifampicin-treated showed them to be completely bacteria-free (See page 130).

Fig. 4.8, shows the cumulative mean egg production of rifampicin and combined rifampicin-tetracycline treated individuals. The patterns of the two sets of data are similar. Cumulative mean egg production reached 55.0 in rifampicin controls and 45.0 to the combined treatment after 7 weeks. Rifampicin and tetracycline treated psocids lived as long as the untreated controls but their egg production especially those fed with rifampicin dropped sharply. This shows that the microrganisms in *L. bostrychophila* ovarioles have a major impact on oocyte development.

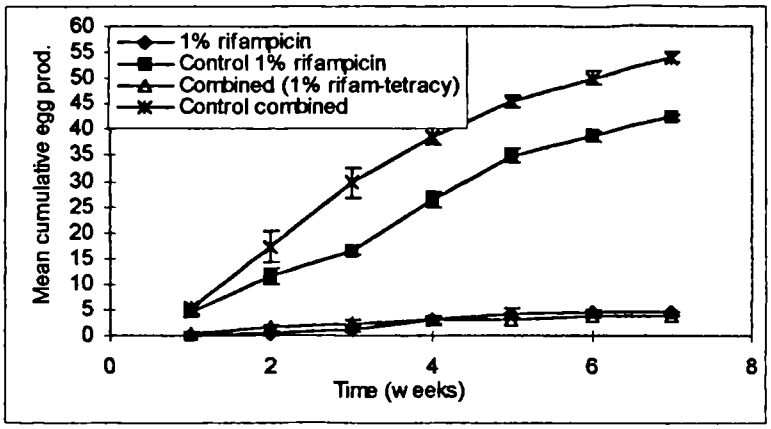


Fig.4.8. Mean cumulative egg production of *L. bostrychophila* females treated with 1% rifampicin or 1% combined rifampicin-tetracycline and their controls.

4.3.3. Adult Mortality

Figures 4.4-6 and 4.9, show the graphic representation of the record adult mortalities in all treatments (heat and antibiotics). Antibiotics, at the dose levels used, did not significantly affect *L. bostrychophila* mortality. Total mortalities during the 7 week experimental period in both treated and untreated adults ranged from 45 to 75 %. Total mortality after 7 weeks, in the combined tetracycline and high temperature treatments reached 75%. Whilst those kept at high temperature, without the antibiotics, suffered a mortality of 60% in the same period.

The mortality levels in the rifampicin treated individuals was not significantly different from that seen in those treated with tetracycline or the high temperature controls. However, the egg output is arrested in the rifampicin treated psocid females after the first week of treatment.

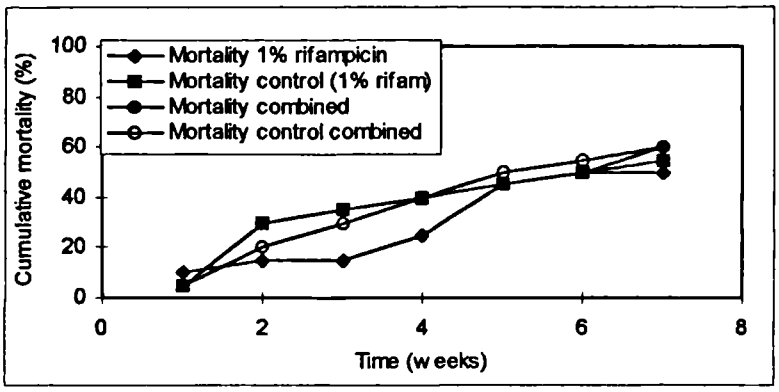


Fig. 4.9. Mortalities of *L. bostrychophila* treated with 1% rifampicin or 1% combined rifampicin-tetracycline and their control.

4.3.4. Ultrastructure of rifampicin treated oocytes

E.M of the rifampicin treated adults (Plate 4.6) confirm the loss of the intracellular bacteria. E.M examination of tetracycline treated *L. bostrychophila* show a reduced bacterial load in the oocytes (Plate 4.2-3 and 4.5). Rifampicin treated *L. bostrychophila* oocytes had no bacteria but showed an increased patchiness and vacuolisation of the cytoplasm (Plate 4.7-8). The patchiness is not however thought to be a negative side effect of the antibiotics, but related to the elimination of bacteria from the oocytes. Heat and tetracycline treated females with extremely reduced bacterial densities also showed the same patchiness and vacuolisation of their oocytes (Plate 4.1-5).

The cytoplasmic vacuoles in the rifampicin treated oocytes begin to fill with large yolk globules (formed from the coalescence of smaller ones) within a week of the start of the antibiotic treatment (Plate 4.8). As shown in Plate 4.6 and 4.8 a drop of electron dense yolk material that appear at the extreme edge of the empty vacuoles, increases in size from 1.5μm until they cover the whole spherical vesicle measuring up to 7μm across (Plate 4.6, 4.8). Rifampicin treated oocytes also showed an increase in the irregularly shaped Protein Lipid deposits (Plate 4.9) which are very electron-translucent and dispersed in the oocyte cytoplasm. Their sizes vary from droplets less than 1.0μm to 8.0μm.

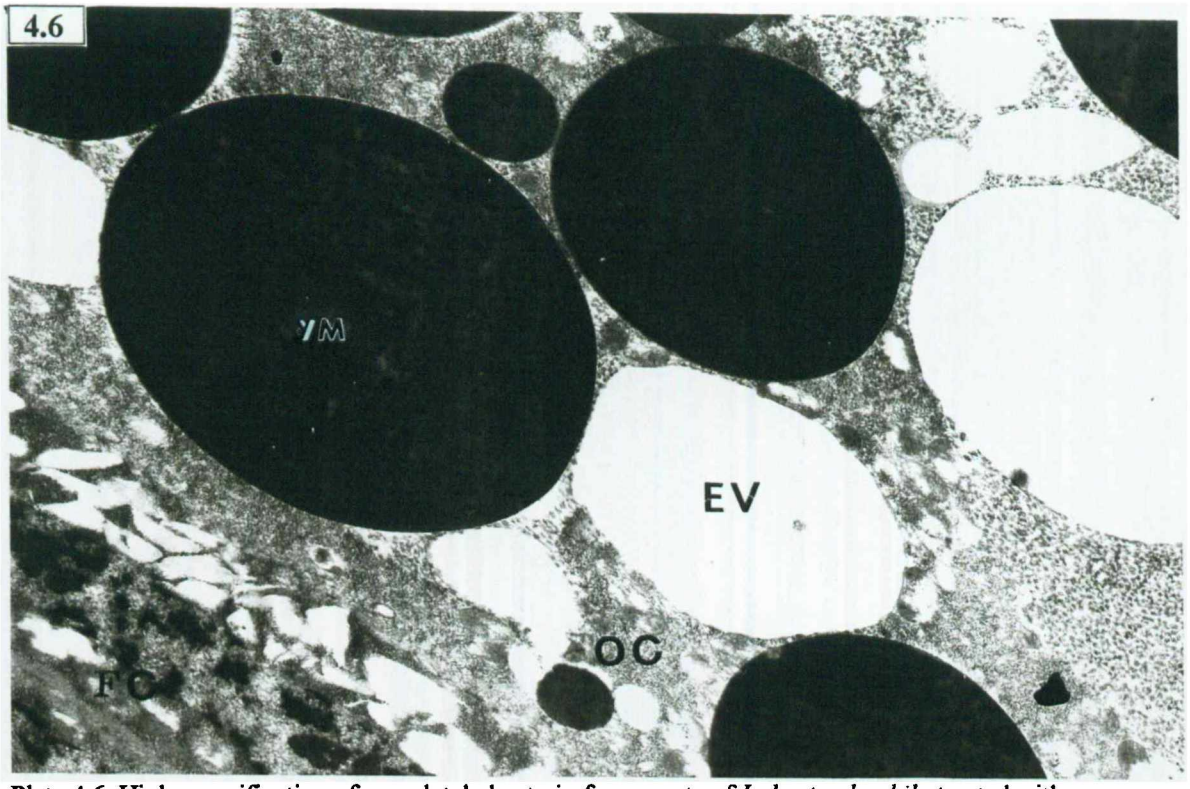


Plate 4.6. High magnification of completely bacteria-free oocyte of *L. bostrychophila* treated with rifampicin. FC, follicle cells; EV, empty vesicles; YM, Yolk material; OC, oocyte cytoplasm Magnification 11600x.

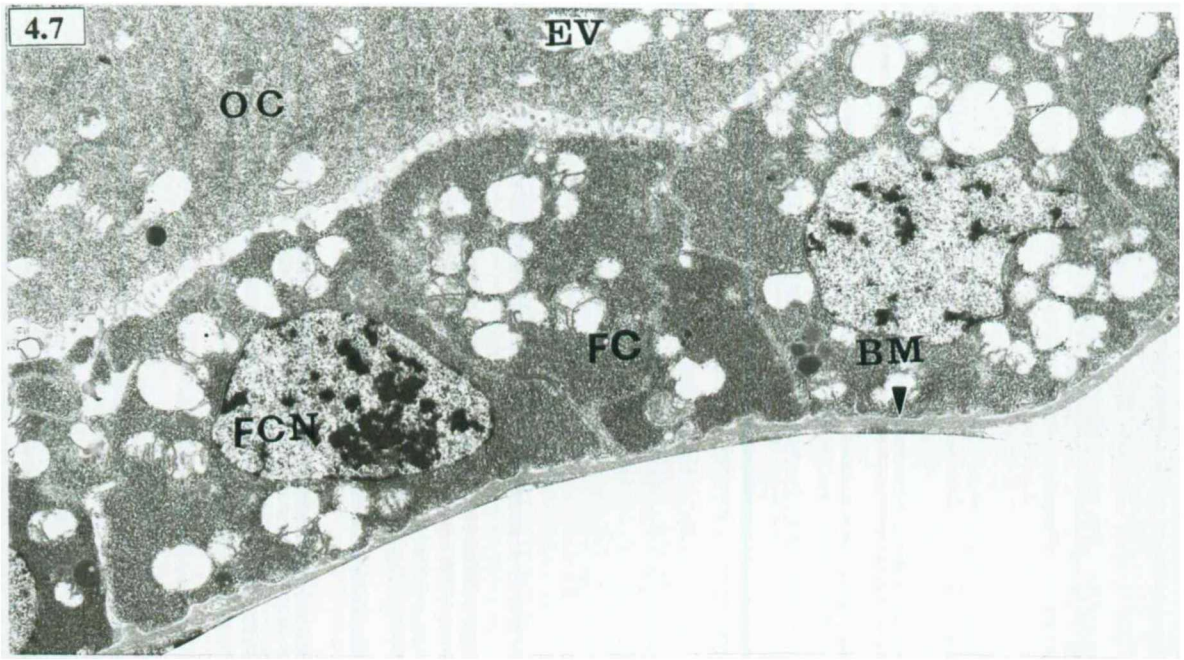


Plate 4.7. Low magnification of rifampicin treated *L. bostrychophila* oocytes showing no bacteria and an increased patchiness and vacuolisation of the cytoplasm. FC, follicle cells; FCN, follicle cell nucleus; EV, empty vesicles; OC, oocyte cytoplasm; BM, basement membrane. Magnification 7200x..

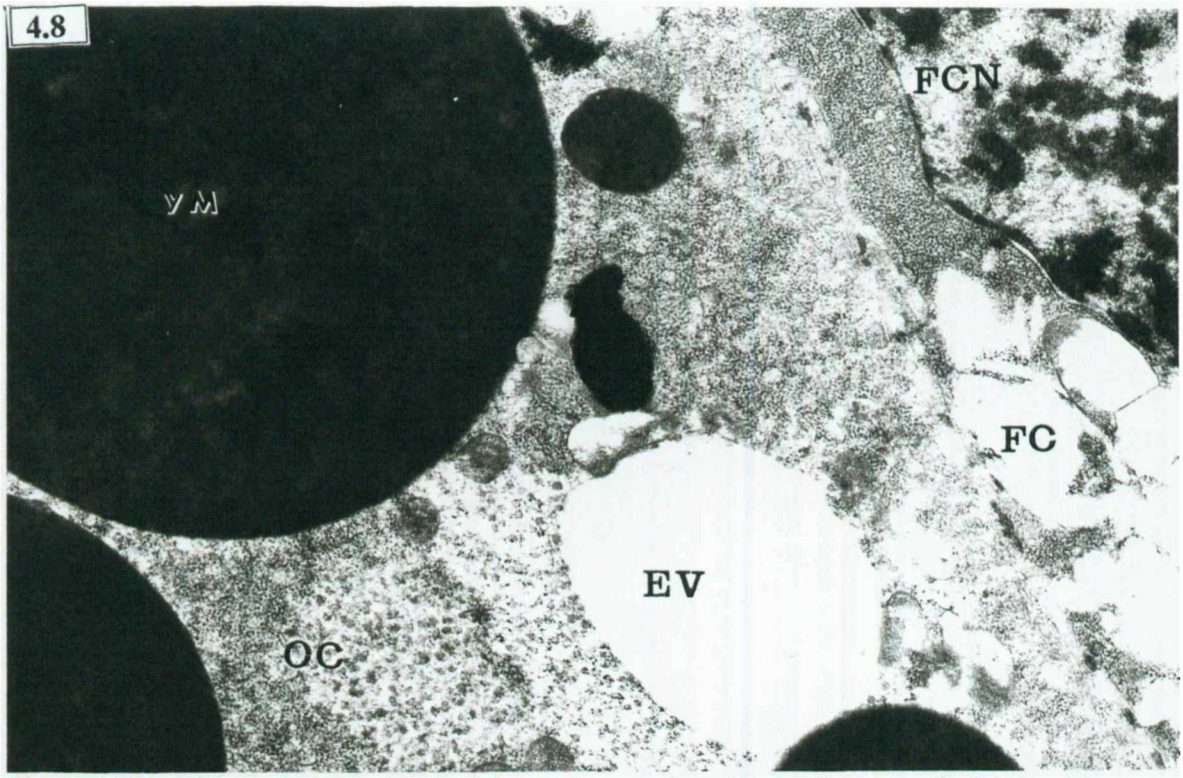


Plate 4.8. Rifampicin treated *L. bostrychophila* oocytes cytoplasm filled with large yolk globules. FC, follicle cells; FCN, follicle cell nucleus; EV, empty vesicles; YM, Yolk material; OC, oocyte cytoplasm. Magnification 20000x.

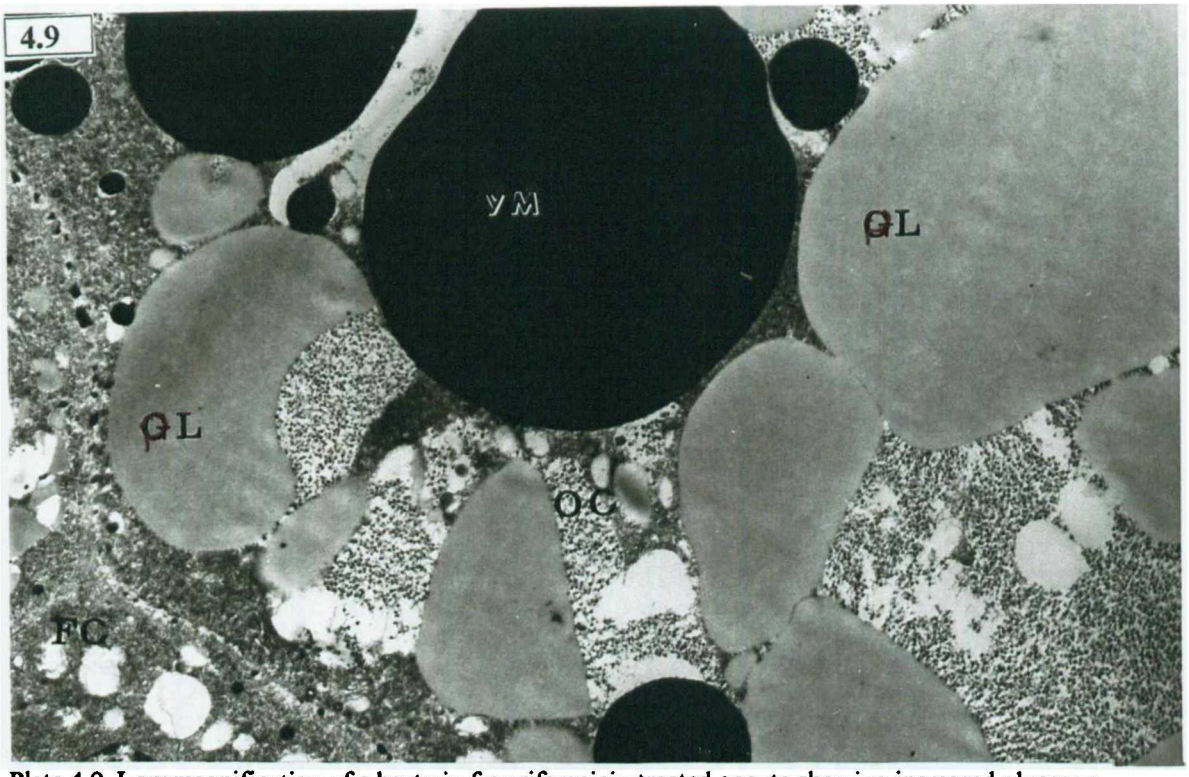


Plate 4.9. Low magnification of a bacteria-free rifampicin treated oocyte showing increased glycogen reserves in less spherical vesicles as well as regularly shaped yolk material. FC, follicle cells; , Yolk material; OC, oocyte cytoplasm; GL/ protein lipid

Magnification 7200x..

4.3.5. Juvenile development

Egg production and juvenile development in treated and untreated *L. bostrychophila* and *L. corrodens* are presented in tables A4.14-16. Treated nymphs took an extra 2-3 days on average to complete juvenile development compared with the controls. Eighty percent (*N=20*) of the 1st instar larvae of *L. bostrychophila* treated with tetracycline become adults compared with the 85% in the controls. Rifampicin treated juveniles had slightly lower survival of 75% (N=20) (Table A4.14).

For the first three weeks the egg production of females treated with tetracycline from hatching was similar to that seen in the controls fed on the standard diet (Plate 4.4). Egg production of the treated individuals dropped to less than half of that of the controls in the third week. The eggs produced by the rifampicin treated females were consistently fewer than the controls from the first week (Table A4.15) suggesting that rifampicin has a more immediate effect in eliminating *L. bostrychophila*'s symbionts. Tetracycline had a more delayed effect on egg production levels.

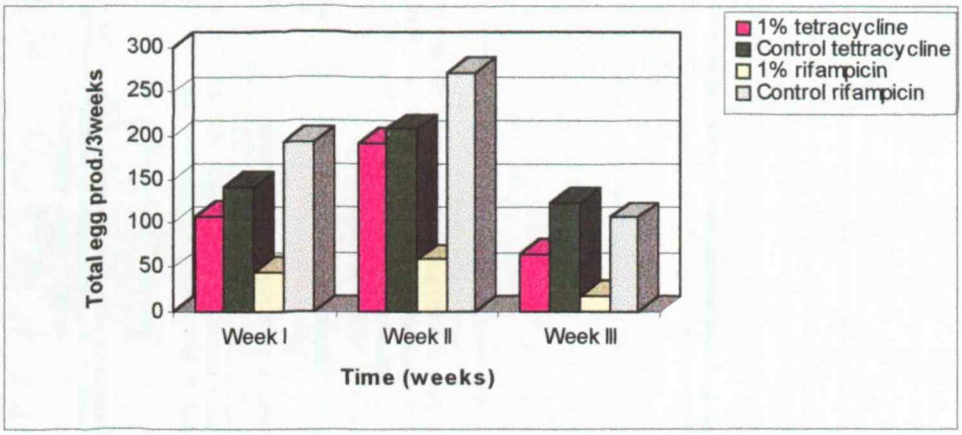


Fig. 4.10. Egg production *L. bostrychophila* adults fed on a diet containing 1% tetracycline and 1% rifampicin during their nymphal stages.

The pattern of egg output of adults treated with antibiotics as nymphs follows a trend similar to that seen in adults fed antibiotics from the commencement of their adult stage (Fig. 4.10).

The difference in egg output between tetracycline treated and their controls were significant. However rifampicin treated individuals are seen to always have a substantially lower rate of egg output through time due to the fact that many adults did not initiate egg production.

Adults treated with antibiotics as nymphs had their egg production monitored for the first 3 weeks. Adult mortalities during these three weeks were relatively low in both treated groups and untreated and ranged from 11-16.5%.

Production of males from treated females was not achieved.

4.3.6. Bisexuals controls

Epifluorescent staining of untreated ovarial tissues of *L. bostrychophila* showed rickettsial inclusion on the peripheries (Plate 4.10). Similar fluorescing bodies were absent from ovaries of the bisexual *L. corrodens* (Plate 4.11).

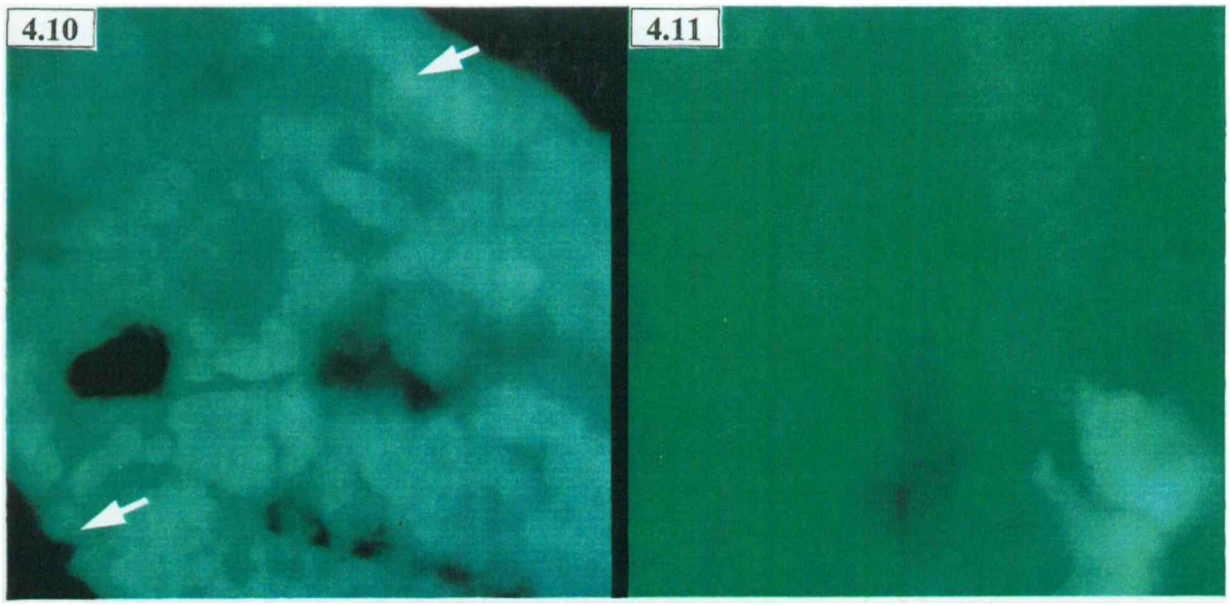


Plate 4.10. Epifluorescent staining of untreated *L. bostrychophila* showing large quantities of small, punctate DNA-positive bodies in the peripheries of the ovarial tissues.

Plate 4.11. Hoechst staining of the ovarial tissues of untreated naturally uninfected bisexual *Liposcelis corrodens*. Fluorescing bodies were absent from ovaries of uninfected bisexual liposcelids.

Antibiotics did not affect their egg production or the longevity and their oocytes did not show any ultrastructural changes.

Egg production of *L. corrodens* is shown in fig. 4.11. Other data including duration of nymphal development and mortalities are shown in table A4.16. Figure 4.11, shows that egg production of *L. corrodens,* when treated with rifampicin or tetracycline, did not significantly differ from the untreated controls although they had a slightly higher egg output. Adult mortalities were also little affected by the antibiotics. Although comparable, the tetracycline treated group suffered a 10% higher mortality than those treated with rifampicin.

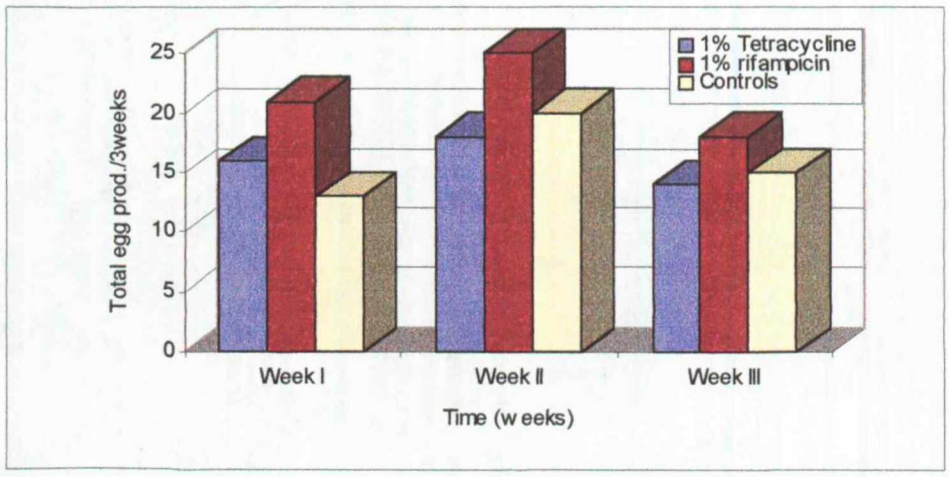


Fig. 4.11. Egg production of *L. corrodens* fed in diet containing 1% tetracycline, rifampicin and their controls.

4.4. DISCUSSION

Aposymbiotic individuals had a very reduced egg output indicating a major but unexplained involvement of the bacteria with the reproductive process. Removal of the bacteria does not have as obvious impact on other systems in *L. bostrychophila* and in particular does not affect the longevity or mortality.

Psocids were reared at 37°C, which should be lethal to the bacteria and close to the threshold temperature for the insect host to survive and reproduce. In some insects the high temperature is not effective because the microrganisms are protected by host membranes and insects are killed before the bacteria can be harmed (Pant and Dang, 1972; Dang, 1971). Ultrastructural investigations of the intracellular bacteria in *L. bostrychophila* revealed no such host membranes. Turner (1994) notes that egg production of *L. bostrychophila* stops around 35°C and it is likely that this is the result of

the destruction of the rickettsiae rather than a true lethal threshold of the insect tissues. Thermal regimes in the tropics might be expected to limit the habitat choice of *L. bostrychophila* in natural conditions to those locations where the temperature is below the lethal limit for the bacteria. Rees and Walker (1990) showed that the bisexual *L. entomophila* was also restricted at 37°C despite not having rickettsiae.

The difficulties in producing aposymbiotic psocids using antibiotics are probably related to the intimate nature of the association and inadequacies in the methods. Because of the small size of *L. bostrychophila*, the only realistic method of administering antibiotics was through the food and this was more successful when the antibiotic was mixed with the food as slurry and then dried. The antibiotics did not show any harmful effects to either the aposymbiotic parthenogens nor naturally uninfected bisexual species. On the contrary the oocytes from aposymbiotic *L. bostrychophila* become structurally similar to those of the bisexual species.

The oocyte of L. *bostrychophila* is organised in highly compartmentalised and mosaic fashion and these large, spherical yolk globules and glycogen vesicles appear in the spaces where the bacteria are found in untreated individuals. All treatments aimed at producing bacteria-free *L. bostrychophila* produced similar ultrastructural changes.

The re-emergence of large yolk components and glycogen reserves in the treated oocytes of *L. bostrychophila* indicates that bacteria modify them structurally in the heavily infected ovaries. The metabolic relationships between the bacteria and oocyte components is unclear. Given the diversity and the abundance of this type of bacteria among insect orders, a wide range of host relationships can be expected to evolve, including mutualisms and infectious pathogenicity (Werren *et. al.*, 1995). Weisburg and co-authors (1989) concluded that the inclusion of the genus *Wolbachia* in the *Rickettsiaceae* link the pathogenic and commensal bacteria that share an ecological niche in the insect body.

CHAPTER FIVE

MOLECULAR CHARACTERISATION OF THE INTRACELLULAR RICKETTSIAL ENDOSYMBIONTS AND THEIR PSOCID HOST *L. BOSTRYCHOPHILA*

CHAPTER FIVE

5.0. MOLECULAR CHARACTERISATION OF THE INTRACELLULAR RICKETTSIAL ENDOSYMBIONTS AND THEIR PSOCID HOST *L. BOSTRYCHOPHILA*

5.1. INTRODUCTION

Chapter 3 describes ultrastructural studies of *L. bostrychophila,* which indicated the presence of endosymbionts in their oocytes which are thought to be *Rickettsia.* Other evidence includes the site of infection, the polymorphic nature of the microrganisms and the inability to culture them in a cell-free media similar to that used for fastidious organisms e.g. Mycoplasma. The inability to grow these endosymbionts in an artificial medium was a serious obstacle to studying these reproductive parasites. The order Rickettsiales contains a collection of bacteria that share the property of an intimate intracellular association with their arthropod host (Weiss, 1984). Only a few *Rickettsia* sp. have been grown in ↖complex host-cell free culture media (Weisburg *et.al.*, 1989). This order contains some notorious pathogens of humans and animals, while some others cause no obvious damage to their hosts and can be considered as commensals (Azad *et.al.*, 1990). The intracellular endosymbionts of *L. bostrychophila* are considered to be in this latter group. Some pathogenic rickettsiae of the genus *Rickettsia* include *Rickettsia typhi* and *R. prowazeki,* the agent of the mild febrile illness called endemic and epidemic Murine typhus respectively. The spotted fever group (*Rickettsia rickettsiae,* the agent of Rocky Mountain spotted fever), and scrub typhus rickettsia *Rickettsia tsutsugamushi* (Azad, 1990) are also important arthropod vectored mammal pathogens. Another group of rickettsiae is *Wolbachia,* which belong to the alpha proteobacteria that infect the reproductive tissues of arthropods. These bacteria are transmitted through the egg cytoplasm and alter reproduction in their insect host in various ways (Werren and Jaenike, 1995). The effects of *Wolbachia* on host reproduction include the ability to induce parthenogenesis.

The relatedness among the rickettsiae (*Rickettsieae, Ehrlichieae and Wolbachieae*) and within the *Wolbachia* group is largely unknown (Weisburg *et.al.*, 1989). For example, good evidence obtained by phenotypic analysis, DNA base ratio determination and

DNA-DNA hybridisation studies have shown that the members of the typhus and spotted fever groups in the genus *Rickettsia*, are related (Weiss, 1984). However the degree of relatedness in these microrganisms to the scrub typhus rickettsia, *Rickettsia tsutsugamashi*, remains unknown (Weisburg *et.al.*, 1989). Similarly, phenotypic analysis links the monocytic Erhlichiae (*Erhlichiae canis, E. sennetsu, and E. risticii*) to each other (Holland *et.al.*, 1985; Ristic, 1986; Ristic and Huxsoll, 1984), but their relationships to the granulocytic Ehrlicheae (*Ehrlichia phagoctophila, E. equi*) and to the endothelial pathogen *Cowdria ruminantium* remains unclear (Weisburg *et.al.*, 1989). Among the *Wolbachia* the relationship between these symbionts and *W. pipientis* has been unclear. The taxonomic position of *W. pipientis* relative to other described members of the genus *Wolbachia* and the phylogenetic position of the Wolbachia in the Eubacteria are also undetermined (O'Neill *et al.*, 1992). These uncertainties are largely due to the fastidious nature of the these bacteria, which have prevented them from being cultured outside their insect host and studied *in vitro* (Krieg and Holt, 1984). The 16S rRNA (encoding for rDNA) is now widely recognised as a conservative macromolecule that allows the phylogenetic placement of these bacterial species (Woese, 1987). It has recently been used to resolve the phylogenetic position of a variety of bacteria placed in the family Rickettsiaceae (Weisburg *et.al.*, 1989; Weisburg *et.al.*, 1991).

The comparison of their rRNA sequences is the most convenient way of deducing phylogenetic and evolutionary relationships among them (Weisburg *et.al.*, 1991). These sequences have been derived previously by methods including oligonucleotide cataloguing (Fox *et.al.*, 1980), cloning and sequencing, direct sequencing of RNA by using reverse transcriptase (Lane *et.al.*, 1985) and sequencing of material amplified by polymerase chain reaction (Boettger, 1989; Edwards *et.al.*, 1989; Medlin *et.al.*, 1988).

The purpose of this part of the study is the isolation and molecular characterisation of the bacterial endosymbionts of *L. bostrychophila.* The data obtained were then used as a basis for determining the phylogenetic relationships between nucleotide sequences of the psocid endosymbionts and published data from other insect species. Variations in 16S sequences from different isolates were also recorded.

5.1.1. Polymerase chain reaction (PCR)

The technology for handling DNA was revolutionised in 1985 with the invention of the polymerase chain reaction (PCR) or thermal cycling by Kary Mullis. The major advance was to develop a method of DNA copying and amplification which could be performed outside the cell (*In vitro*). The PCR method is outlined in Fig. 5.1.

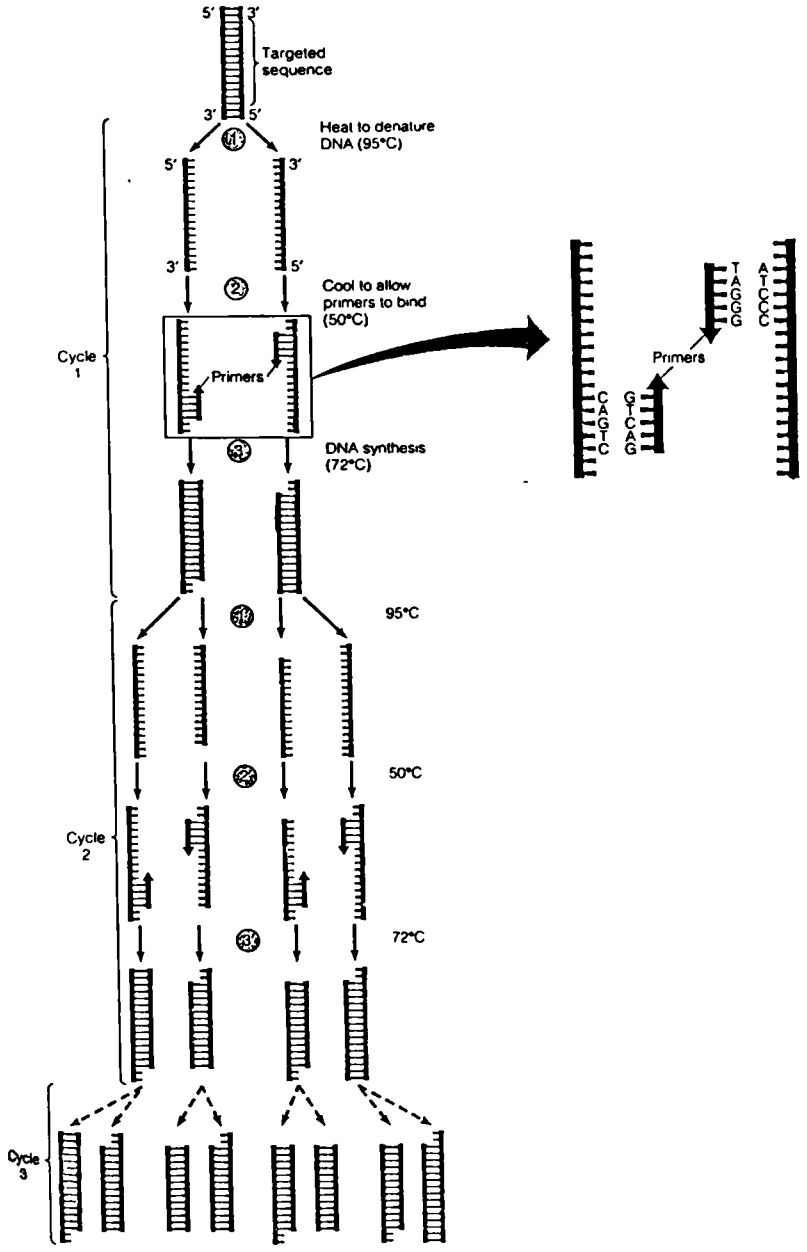


Fig. 5.1. The technique of polymerase chain reaction (Robert J. Brooker, 1999).

PCR uses simple enzyme reactions and the amplification is specific, defined by primers. To start the reaction two oligonucleotides (or primers), short pieces of DNA, normally 20-40 base pairs, are synthesised corresponding to the DNA sequence on either side of the stretch (in this case 16S rDNA) to be amplified. The starting DNA molecule is heated strongly so as to separate its two strands (Denaturing) and then cooled in the presence of oligonucleotides (Fig. 5.1). These complexes of short DNA with long, single stranded DNA are then able to 'prime' the synthesis of more DNA by the polymerase enzyme which adds all four nucleotides (ATC and G) to the 3'[1]- end of each short DNA, until the 5'- end of the long DNA is reached (Annealing). By base pairing, the polymerase makes a new, full length double helix from each of the two strands which were present in the mixture after heating.

Once the primers have annealed, *Taq* polymerase (a thermostable form of DNA polymerase isolated from *Thermus aquaticus*) will catalyse the synthesis of complementary DNA strands. This doubles the amount of template DNA. The sequential process of Denaturation-Annealing-Synthesis is then repeated many times giving an exponential increase in the amount of template DNA. The method is called a chain reaction, because the products of each previous reaction (i.e., newly made DNA strands) are used as reactants (i.e., as template strands) in subsequent reactions. The PCR is carried out in a thermal reactor that automates the timing of each cycle. The DNA sample, primers, *Taq* polymerase and deoxynucleotides are mixed together in a single tube. The tube is placed in the thermocycler to operate within a defined temperature range and number of cycles.

PCR has rapidly acquired a core role in research because of its simplicity, speed, ability to tolerate low grade substrates, and its sensitivity (it has made DNA analysis possible on minute samples).

5.1.2. Approaches to bacterial characterisation

The 16S r-RNA (r-DNA) as a conserved bacterial gene is used for genetic characterisation, placement and the phylogenetic analysis of bacterial endosymbionts,

[1] **The first carbon atom, the 1′ carbon, is by definition the carbon atom covalently attached to one of four organic bases: (A, G, C, or T). Phosphate groups are attached to the third (3′) and fifth (5′) carbon atoms.**

without the necessity of culturing the bacteria independently of the host. The nature of these endosymbiont rickettsiae was investigated using PCR to amplify this gene. The PCR product was then characterised by restriction and sequence analysis. Computerised databases of complete 16S rRNA sequences exist for a wide range of bacterial taxa for comparison.

The amplification of 16S rDNA has also been used to develop a bacterial bioassay for determining the infection status of infected and treated (antibiotic and heat) individuals of *L. bostrychophila* populations and naturally uninfected bisexual liposcelids.

5.1.3. Psocid genetic characterisation

The 12S gene encoding for the mitochondrial rRNA of insects is a widely used genetic marker. This gene, although highly conserved, shows single base variation that is important for typing purposes. The sequence analysis of the subunits of the rRNA in mitochondrial DNA (mtDNA) is suitable for studying the evolutionary rate of variations between closely related insect subgroups (Simon, 1991). Sequence relationships of the small (12S rDNA) coding region in the mitochondrial DNA within and between populations of *L. bostrychophila* have been analysed.

Since all individuals of *L. bostrychophila* are completely parthenogenetic, a single psocid can start an entire population. The population can then become widely distributed over relatively large area, carried mainly by human activities. Determining nucleotide sequences would allow two geographically distant psocid strains to be classified as possible close relatives, by obtaining the insect and the bacterial isolate associated with it. Some bacteria show a concordance in phylogeny based on the 16S rDNA gene and the phylogeny of the host insect, as observed in aphid-symbionts *Buchnera aphidicola* complex (Moran *et.al.*, 1993). To show any such concordance between the intracellular endosymbionts and their psocid host, the 16S rDNA and 12S genes were needed to be sequenced simultaneously.

The 12S subunit of the psocid host is shorter with a length of about 400 base pairs and is easier to sequence.

5.2. METHODS AND MATERIALS

5.2.1. Template (DNA) preparation, isolation and purification

Two methods of DNA extraction (Proteinase-K and DNAce) were used. The Proteinase-K method is simple and inexpensive but produces impure DNA. The DNAce silica based technique was later adopted as the routine extraction method because it provided relatively large amount of DNA and was more successful in the PCR amplification and yield of the amplified product.

5.2.2. Proteinase-K extraction

Psocids (10-100 individuals) were surface sterilised in a series of distilled water and 70% ethanol washes prior to the DNA extraction. Insect ovarial tissues were dissected in 50μL of STE (100mM NaCl/ 10mM tris Cl, pH 8.0/1mM EDTA). The tissues and the solution were homogenised using a DNA-free polypropylene pestle. Homogenised psocid tissues were then incubated with 2μl of Proteinase-K (10mg/ml) for 30 minutes at 37°C, followed by 5 minutes at 95°C for denaturing. The sample was spun in a microcentrifuge for 5 minutes to pellet the DNA. 1-2μl was then taken for amplification.

5.2.3. DNAce clinipure purification system (Bioline)

In this method the DNA is bound with a silica solution and is used for large scale genomic extraction of fresh tissues. As recommended by the manufacturer, 0.5-20 mg of surface sterilised psocid tissues were homogenised in 500μl of lysis buffer D and incubated at room temperature for 5 minutes. The sample was then centrifuged in a microfuge at top speed for 5 minutes and the clear supernatant removed to a fresh tube. 15μl of silica suspension was added to the supernatant and mixed by manually inverting and then incubated on ice for 5 minutes. This mixture was centrifuged at 10K for 5 seconds to pellet the DNA and the supernatant was discarded. The pelleted DNA was redissolved by adding wash buffer and incubated on ice for at least 5 minutes. After washing, the silica-bound DNA was microfuged at top speed for further 5 minutes and the DNA was pelleted again and dried by placing the open tube containing the DNA in

a 52°C water bath. The DNA was then eluted with 50µl elution buffer (water) for 5 minutes and stored at 20°C.

To monitor the abundance of the extracted DNA and the size of the subsequent amplification a fixed proportion (1/10) was always loaded onto a 1.5% agarose gel, electrophoresed at constant current for 2 hours, stained with Ethidium Bromide and examined under the UV light.

Extreme care was taken throughout the procedure to avoid contamination. Sterile solutions, distilled and sterile water and aerosol resistant tips (ART) were used routinely. The gel electrophoresis was carried out in plastic tanks containing 25Mm TBE buffer containing EtBr at 60-80V.

5.2.4. PCR amplification and isolation

The 16S r-RNA bacterial gene was selectively amplified from the total genomic DNA extracted from insect ovarial tissues, using generic and rickettsial specific primers. The details of the primers used for the amplification of bacterial 16S and insect 12S genes are displayed in Table 5.1.

These primers are specific to the 16S rDNA gene and used for classification and genetic characterisation of rickettsiae and other Eubacteria (Weisburg *et al.*, 1991). O'Neill and co-workers (1992) and Weisburg and *et.al.*, (1991) described primers for *Wolbachia* strains and wide ranging Eubacteria respectively. Their primers were used successfully for the enzymatic amplification of bacterial 16S rRNA (rDNA) of psocid endosymbionts.

Other internal primers that amplify the 16S rDNA into small subunits were designed. The 16S rDNA macromolecule is approximately 1.5 kb (1460 base pairs) and was divided into three smaller subunits of roughly 500 base pairs each. Forward and reverse internal primers running from the last 5-10 bases of the last subunit were designed from the initial 16S sequence obtained.

Primers	Sequences	Position	Target	Size of product	Cycling condition	Reference
16SF 16SR	5'- GCT TAA CAC ATG CAA G 5'-CCA TTG TAG CAC GTG T	41-61 *E.coli* 1242-1227 *E.coli*	Eubacteria	1180 bp	95(1min), 55 (1min), 72°C (14min) for 30 cycles	O'Neill *et.al.*, 1992
fD1 rD1	AGA GTT TGA TCC TGG CTC AG AAG GAG GTG ATC CAG CC	339-357 357-342	*Rickettsia/ Wolbachia*	1450 bp 1450 bp	95 (1min), 42 (30 sec) and 72°C (4min) for 30 cycles	Weisberg *et.al.*, 1991
16SI-I 16SI-II	5'-CCG CGG TAA TAC GGA GAG GCG TAG 5'-CCT CAGE CAC GEC TAG ACC AC	500-100. 16SrDNA gene 1000-1500 16SrDNA gene	Internal 16S primer Internal 16S primer	1500 bp 1500 bp	95 (1min), 42 (30 sec) and 72°C (4min) for 30 cycles	Designed by the author Designed by the author
12SF 12SR	5'AAA CTA GGA TTA GAT ACC CTA TTA T 5'-AAG AGC GAC GGG CGA TGT GT	Insect 12S gene Insect 12S gene	Insect 12S gene	400 bp 400 bp	95 (1min), 42 (1min), 72°C (1min) for 30 cycles	O'Neill *et.al.*, 1992

Table 5.1. The amplification primers used for the genetic characterisation of *L. bostrychophila* and its rickettsial endosymbionts.

The primers (12SAI and 12SBI) used in amplifying the small 12S rDNA of insect hosts were described by O'Neill *et.al.*, 1992.

The amplification of the 12SAI and 12SBI were also used as controls to check the quality of DNA extraction in both Proteinase-K and DNAce clinipure purification systems.

5.2.5. Gel Purification

DNA isolated in the agarose gel was cut out and purified using standard ethanol precipitation. 25mM TBE buffer containing DNA was mixed with equal volume of phenol to precipitate non-DNA material. This mix was centrifuged for 1 minute and the supernatant solution containing DNA was measured with pipette and put it in a new tube. The DNA was then precipitated using 10th volume of 3M NaAc salt and 2.5 times the volume of absolute alcohol. This solution was manually homogenised and chill dried for less than 5 minutes. The DNA containing solution was then centrifuged for 5 minutes to pellet the DNA on the side of the tube. The salt and alcohol containing

supernatant was discarded. The remaining liquid was drained completely using paper tissue. The DNA pellet was then washed with 70% ethanol, centrifuged for 2-5 minutes and the alcohol removed. The remaining alcohol was further vacuum dried for 5 minutes. Clean, purified DNA pellets were redissolved in 10µl of distilled, sterile water. Oneµl of which was mixed with 2µl of loading dye and 7µl of water and loaded into agarose gel to verify the quality of the DNA extraction.

5.2.6. PCR optimisation

The PCR was optimised under a given temperature cycling conditions by varying the amount of MgCl put in the reaction. Magnesium chloride concentrations of 1, 1.5, 2 and 2.5mM were tested. Temperature conditions and the number of cycles were extended or reduced until a satisfactory limit was found. Only poor results were further optimised until a satisfactory amplification was achieved.

Approximately 1-3µl of the extracted DNA was used as template for amplification in a total volume of 100µl of reactant (25µl/tube). The PCR reactants mix contained 10µl 10x PCR buffer (Promega), 10µl of nucleotide mix (2 mM each), 5µl of 10µM primer 1 (Forward), 5µl of 10µM primer 2 (Reverse), 20µl MgCl 7.5mM, 5u/µl *Taq* polymerase (Promega) and double-distilled, sterile water added to give a final volume of 100µl.

PCR cycling was conducted using a Techne thermal reactor PHC-2 and Uno-thermoblock (Biometra) thermal cyclers. Negative controls of rifampicin-cured populations and naturally uninfected species were used to ensure that only the target DNA was amplified and not that of other contaminant bacteria.

Target DNA sequences, amplified by PCR, were detected by EtBr staining after agarose gel electrophoresis. For gel analysis 10µl of each PCR product was loaded in 1.5% agarose gel, calibrated with standards of known molecular size.

5.2.7. Cloning the PCR product

16S and 12S rDNAs genes from 15 populations of *L. bostrychophila* were cloned. Cloning of the PCR product was carried out using standard methods (Maniatis *et al.*, 1982). PCR products were ligated into a plasmid vector to produce a relatively large amount of sequenceable DNA.

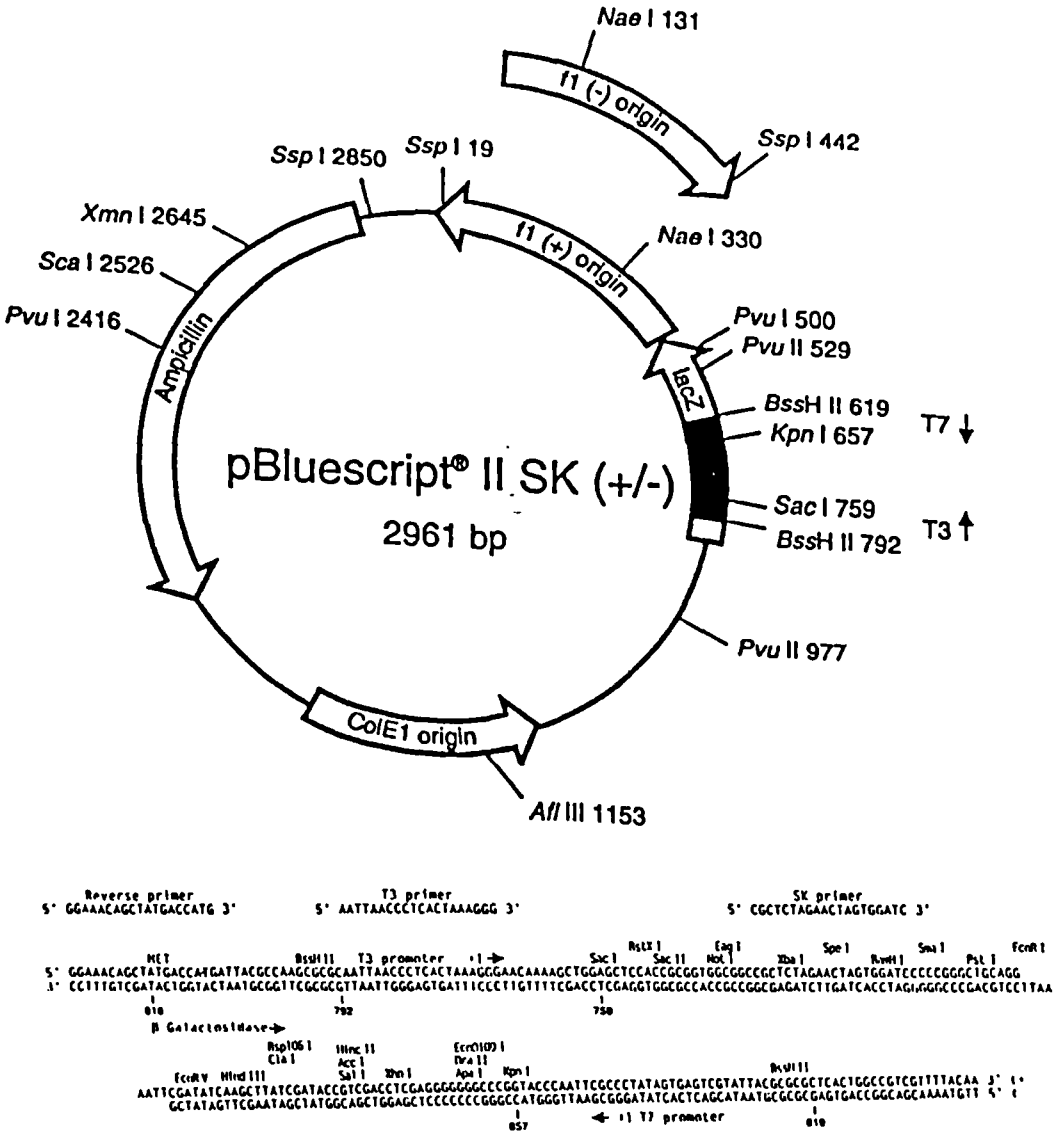


Fig. 5.2. Circular map of the pBluescript ® II (+/-) phagemid used as vector. pBluescript is 2961 bp long and provided successful clones for both insect and bacterial genes.

Ligation: Amplified 16S rDNA fragments were ligated to a pGEM-T® or T-tailed pBS vector (Fig. 5.2). The ligation was carried out in 10μl reactions containing 50μg of the vector, 2μl of the PCR product, 1μl of ligation enzyme and the presence of 1μl of 10x ligation buffer (Promega). This was added to 5μl of sterile water to make up the 10μl reaction. The reaction was left in room temperature overnight. The PCR product of the *L. bostrychophila* symbionts were cloned into the *LacZ* site in this manner (Fig. 5.2).

Transformation: The transformation of the ligated fragments was achieved by adding 40-50μl of XL1-Blue competent cells. This mixture was then left on ice for 30 minutes. This was heat shocked at 42°C for 2 min and put back into ice. 200μL of LB (liquid bacterial broth) is added and shaken for 1 hour at 37°C. Bacteria colonies containing competent plasmid were then plated out on Amp/X-Gal plates and incubated overnight at 37°C. Recombinant colonies coloured white by interfering with the *LacZ* gene while the parental colonies coloured blue. Twenty white colonies were randomly picked, half of them were added to 20μl water in 1.5ml tubes and boiled at 95°C for 2 min to denature the content and centrifuged for 5 min to precipitate the cell debris. One μl of the supernatant DNA was put in 20μl PCR reaction and using relevant primers for 16S rDNA gene amplified.

The other half of the selected white colonies were minipreped to produce large amounts of DNA. Each colony was suspended in approximately 4μl of LB with ampicillin and left to grow overnight at 37°C. The cultures were briefly centrifuged to pellet the cells and then resuspended successively in 250μl of solutions I, II, and III for cell lysis and to denature RNAse respectively. The solution was then centrifuged for 5 min, transferred to tubes and heated in a water bath (50-60°C) for 30 min to denature the enzymes. The DNA was then phenol chloroform/isopropanol extracted and washed with 70% ethanol, pelleted, vacuum-dried and redissolved in 25μl of water to store frozen. Some 'parental' blue colonies were also processed as a plasmid control.

5.2.8. DNA restriction analysis

The amplification of the 16S gene produced a PCR product of approximately 1.5KB. Cloned (recombinants) and parental colonies were also restricted and the released DNA

fragments analysed. Restriction digestion was done in a 10µl reaction containing 1µl of the target DNA (plasmid or direct PCR product); 1µl restriction buffer, 1µl restriction enzyme, and 7µl of water were added to make a total 10µl reaction. The restriction reaction is incubated at 37°C overnight. The restriction enzyme used was XbaI for amplified rDNAs which produced two characteristic *Rickettsia* DNA fragments (Werren *et.al.*, 1994). EcoRI, and NotI were also used to restrict the target DNA carried by the plasmid vector. The restriction enzymes were useful in assessing whether or not successful cloning of 16S rDNA into the plasmid vector had been achieved. Ten µl of the restriction product was loaded into agarose gel for observation. Uncoiled and linear DNA with the right size fragments was released in successful clones.

5.2.9. Sequencing and phylogenetic analysis

Sequencing was done using ABI PRISM ® 377 automated cycle sequencer (Perkin Elmer) with each terminator fluorescence labelled with a different colour. Ten µl of a reaction solution containing 2µl of template DNA (100ng/µl) of very clean Plasmid DNA, 4µl pre-mixed sequencing reagents (*Taq*, dNTPs and ddNTPs), 2µl of each primer and 2µl of water was prepared.

Single strand cycle sequencing was achieved using SP6 (Forward)/T7 (Reverse) pGEM and T4 (Forward) / T3 (Reverse) primers in pBluescript (pBS). Respective amplification primers were also used where PCR products were directly sequenced. The cycling conditions were 96°C for 20 seconds, 56°C for 20 seconds and 60°C for 2 minutes.

DNA was then precipitated with Sodium acetate and absolute ethanol, washed using 70% ethanol and vacuum-dried. The DNA was then redissolved in 4µl of loading dye (red dye). Redissolved DNA was denatured briefly at 95°C for 2 min. Samples were loaded on to a pre-prepared acrylamide gel and the sequence reactions were performed in Biometra UNO-thermoblock.

The sequences generated were reformated using a Wisconsin Package Version 9.1, of the Genetics Computer Group (GCG), Madison, Wisconsin. USA.

Sequences were then introduced into a sequence editor (Chopup) and aligned against collections of 16S rDNA sequences from various entomogenous bacteria.

The degree of similarity between homologous sequences was then determined by using GCG programmes of pileup, plot similarity, plot similarity -iden and figure. These software are used to align multiple sequences and dendograms were constructed to show the evolutionary relationship between *L. bostrychophila* endosymbionts and other representative members of arthropod-infecting, α-Proteobacteria. Sequences from different populations of psocids were also aligned and their variations examined.

5.3.0. RESULTS

5.3.1. PCR bioassay as a diagnosis for rickettsial infection in *L. bostrychophila.*

Proteinase-K extracted genomic DNA tended to be impure or low in DNA content and needed a more rigorous optimisation. This technique was less sensitive and required a relatively large quantity of homogenised psocid material. Proteinase-K extraction routinely yielded detectable levels of bacterial rDNAs around 20ng/λ ($\equiv 20ng^{-1}\mu l^{-1}$)

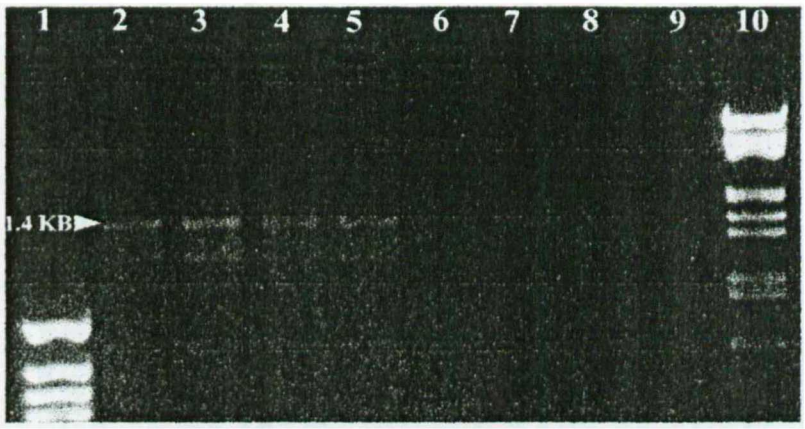


Fig. 5.3. PCR product of Proteinase-K extracted template (Lanes 2-5). The amplified DNA (approximately 20 ng/λ), enough in detecting the bacteria but below the concentration required for sequencing. Rifampicin treated lines (6-9) show no amplification.

(Fig. 5.3). The results were consistent with that of the other technique; for example no amplification products were produced in bacteria-free Rifampicin treated individuals (Fig. 5.3). The DNAce protocol, using the same conditions (MgCl concentrations, cycling and heating) as that used for Proteinase-K extracts, produced relatively cleaner and higher yield of DNA. The amount of amplified DNA ranged from approximately 40 to 80ng/λ of symbiont rDNAs (Fig. 5.4).

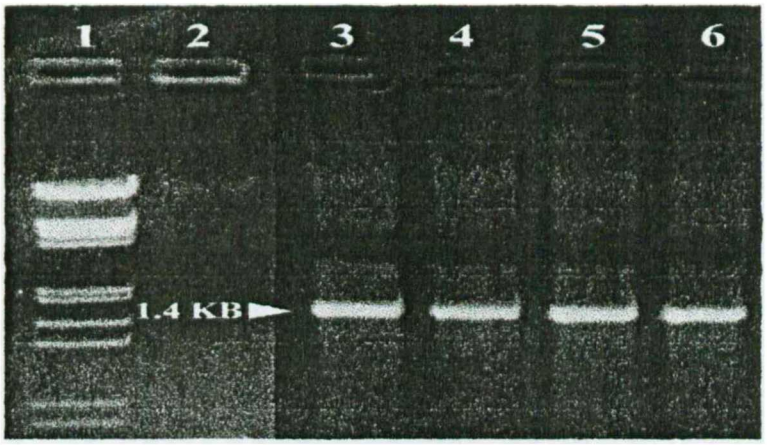


Fig. 5.4. Rigorously optimised PCR product using DNAce clinipure purification system extracted as template (Lane 3-6). This provided larger and cleaner DNA in comparison to the simpler Proteinase-K extraction technique.

All populations of *L. bostrychophila*, screened using these methods, harboured detectable levels of rickettsial DNA. Successful PCR reactions amplified fragments of 16S rDNAs (approximately 1.5 KB long) from the infecting bacteria (Fig. 5.5). Of the 30 liposcelid populations screened by this method, 15 were successfully cloned, and of these just over half provided sufficiently clean sequence data for multiple sequence comparisons.

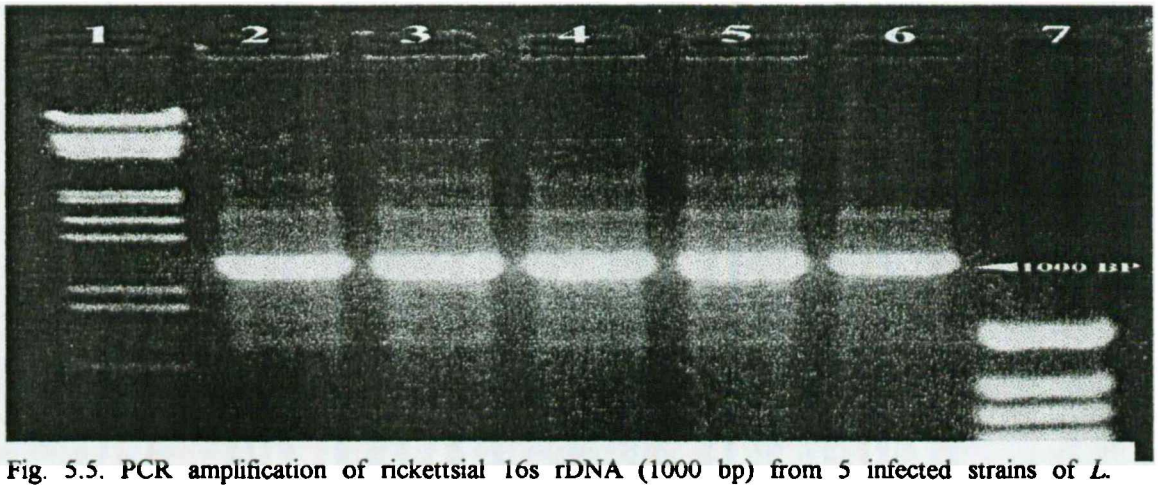


Fig. 5.5. PCR amplification of rickettsial 16s rDNA (1000 bp) from 5 infected strains of *L. bostrychophila* using 16SF/R general primers (lane 2-6). Lane 1 (Lambda/EcoRI/Hind III) and 7 (pBluescript II SK+ -Hpa II Digest) are 10µl of standard weight markers.

Rifampicin-treated individual psocids were also tested using PCR assay to determine if they harboured detectable levels of endosymbionts. These psocid lines become uninfected and tested negative confirming that the bacteria were lost during the five week long treatment (Fig. 5.6).

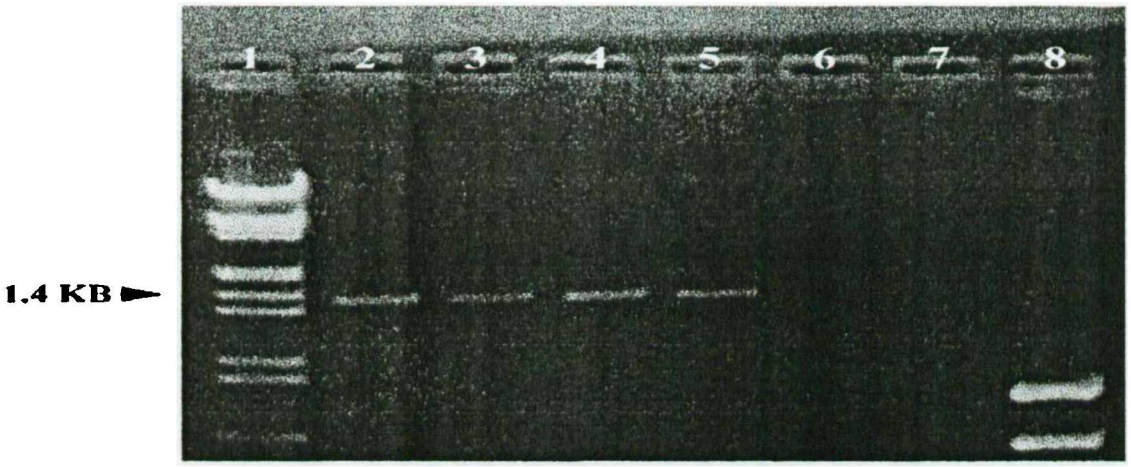


Fig. 5.6. The 16s rDNA amplification from 4 infected *L. bostrychophila* strains (Lane 2-5) and two aposymbiotic, bacteria free lines (Lane 6-7) showing no amplified product. Amplification primers were fD1 and rD1 and genomic DNA was extracted using silica-based DNA Clinipure (Bioline).

The PCR amplification of genomic DNA extracted from the reproductive tissues of the closely related bisexual *Liposcelis corrodens* did not show the presence of any rickettsial DNA (Fig. 5.7), and support the microscopical evidence of the absence of endosymbionts in this species.

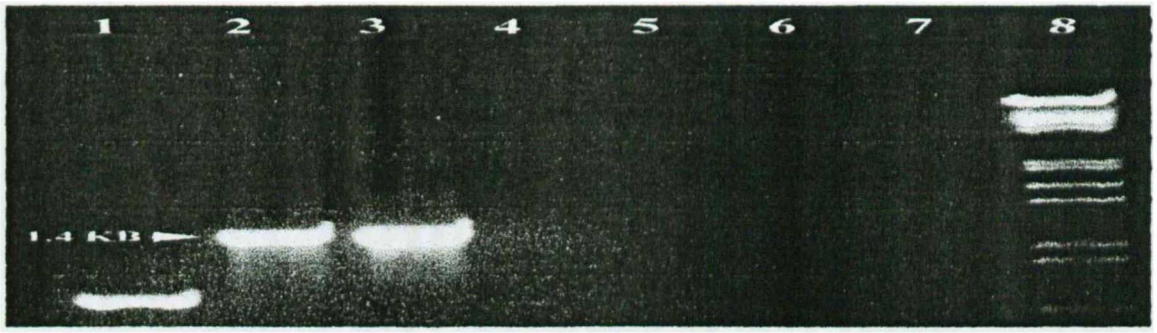


Fig. 5.7. PCR assay for *L. bostrychophila* infection using *Rickettsia* specific primers (Lane 2-3) and 4 samples from the bisexual *L. corrodens* showing no infection. Genomic DNA was extracted using silica-based DNA Clinipure (Bioline).

5.3.2. Cloning results and restriction analysis

Large scale PCR amplification was performed on DNAce clinipure extracted genomic DNA, which contained both bacterial 16S and insect 12S rDNAs. PCR products of amplified 16S rDNA from *L. bostrychophila* using rickettsial primers (fD1 and rD1) provided a satisfactory template for cloning (Fig. 5.8). Figure 5.8, shows 10µl of such amplification in 11 populations of *L. bostrychophila*. Psocid lines that have yielded relatively large amount of DNA were isolated, electro-eluted and phenol precipitated.

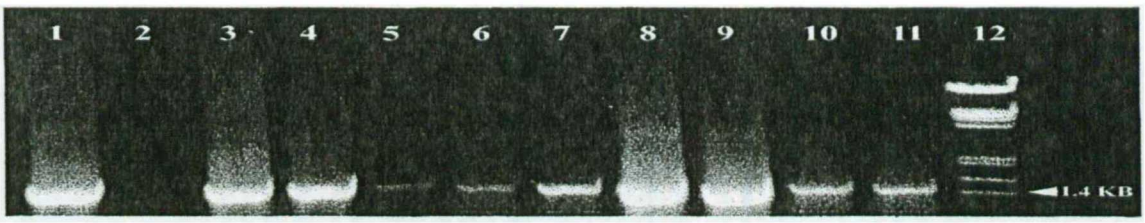


Fig. 5.8. Large scale PCR screening of bacterial infection from 31 populations of *L. bostrychophila*. This gel shows 11 populations that all tested positive (Lane 1-11), Lane 12 is standard marker (Lambda/EcoRI/Hind III). This DNA is gel purified and cloned.

This DNA is then ligated to the pBS plasmid vector, transformed into competent bacteria and cloned. The PCR amplified DNA was then used for identification purposes.

Fig. 5.9, shows the PCR products of the plasmid vector carrying the inserted 16S rDNA gene of the rickettsial endosymbionts from different populations of *L. bostrychophila* (Lane 1-5). In lane 6-16, show no insertion and cloning has failed. Two clones with the right fragment size per population (e.g. Lane 4 and 5) were chosen and minipreped in each instance. Occasionally some colonies provided products of the wrong size (Fig 5.10, Lane 4). In such cases these chiamera were discarded and two samples with same size as the target were selected from the multiple colonies for further analysis.

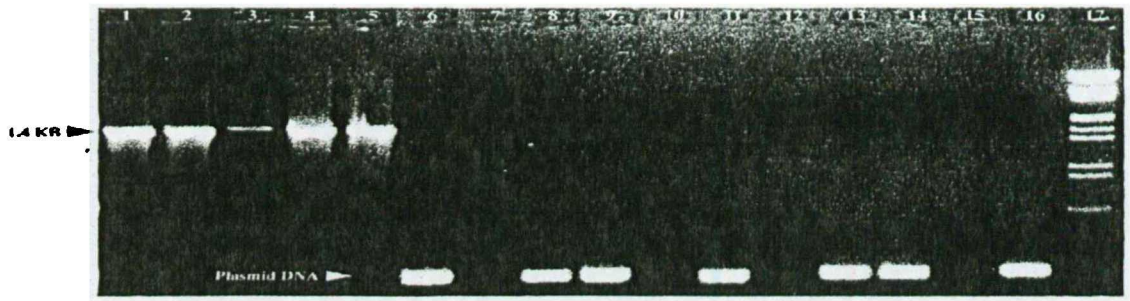


Fig. 5.9. PCR determination of successful cloning of the 16S bacterial gene inserted into the plasmid vector (Lane 1-5). Plasmid carries no target DNA in other lanes (Lane 6-16). Two clones (e.g. Lanes 4-5) were selected per population. Lane 17 is pBS II SK+ -Hpa digest marker.

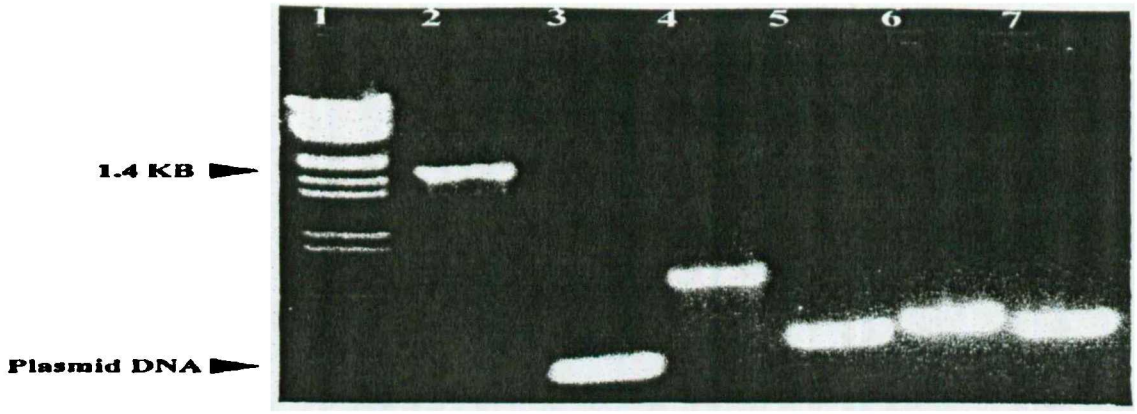


Fig. 5.10. Agarose gel showing occasional errors that occur in the cloning process whereby clones with wrong size product (Lane 4) than the target (Lane 2) is amplified. Since only one clone carrying the right fragment size was amplified in this instance, this DNA (lane 2) was not used. Lane 3, 5, 6, and 7 cloning failed. Lane 1 is Lambda/EcoRI/Hind III marker.

The sequenceable rDNAs were boosted by cloning and a large quantity of supercoiled plasmid DNA was produced (Fig. 5.11). The amount of DNA produced varied from 1.0mg (1000ng)-2.5mg/λ. These DNAs were diluted to approximately 100ng/λ for sequencing.

The restriction profiles of the 16S rDNAs were determined from directly amplified products as well as clones with right fragment-size insertion. The restriction enzyme

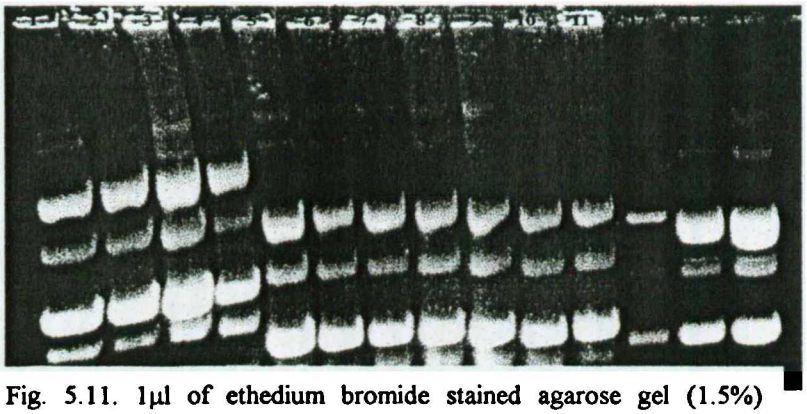


Fig. 5.11. 1μl of ethedium bromide stained agarose gel (1.5%) showing supercoiled plasmid DNA carrying the bacterial 16S rDNA gene (Lane 1-11). DNA is boosted to up to 2.5 mg/λ, diluted accordingly for multiple sequencing. Lane 12-14 standard pBluescript marker weighing 200, 1000, 2500ng/λ.

XbaI gives a characteristic pattern for *L. bostrychophila* endosymbionts and other insect-infecting rickettsias. XbaI cuts the 16S PCR product of the *L. bostrychophila* endosymbionts once, giving two fragments of 559 and 876 bp in length (Fig. 5.12). This restriction enzyme was used to determine whether the PCR product and clone inserts contained the expected 16S product and whether the PCR amplifications were homogeneous or heterogeneous for the 16S product.

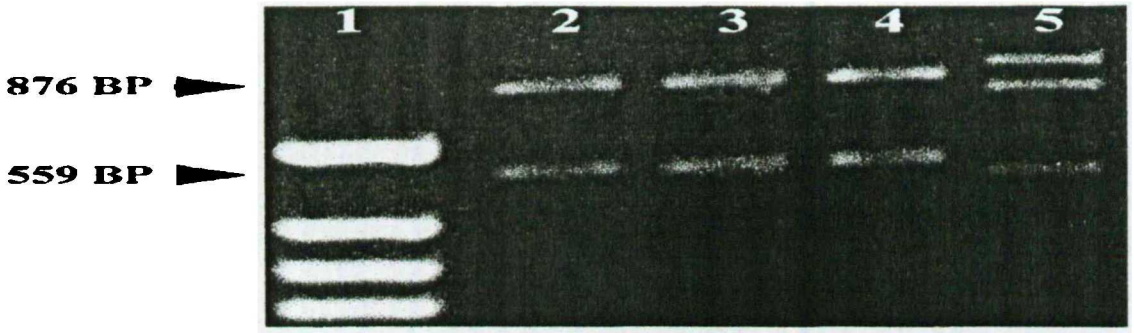


Fig. 5.12. The restriction product of the amplified rickettsial 16S DNA using XBA1 produced two fragments sized 559 and 876 bp (Lane 2-4) characteristic for rickettsiae. Lane 1 and 5 are standard size markers.

5.3.3. Bacterial 16S rDNA sequence analysis

The cycle sequencer (ABI PRISM 377) provides high throughput and an automated base calling and data collection. Figure 5.13 shows a typical output in which the sequential presence of different bases are indicated by the different coloured graph plots and interpreted as a sequence given above the plots.

Since small amounts of sample are increased by PCR amplification, it is less faithful in DNA copying than biological replication. It is therefore possible to confuse errors made in PCR copying (*Taq* polymerase infidelity) with genuine biological sequence differences. To overcome these difficulties inherent with PCR, the total PCR product was sequenced yielding a consensus of sequences. However, PCR products were not pure or in large enough quantities on all occasions, so cloning offered an attractive alternative. To provide a check on the results two clones were sequenced from each psocid population: if the two sequences agreed they were considered correct, but where they disagreed the base was not called as it may have mutated during PCR (*Taq* polymerase errors). In this case a third consensus sequence is performed or the process repeated.

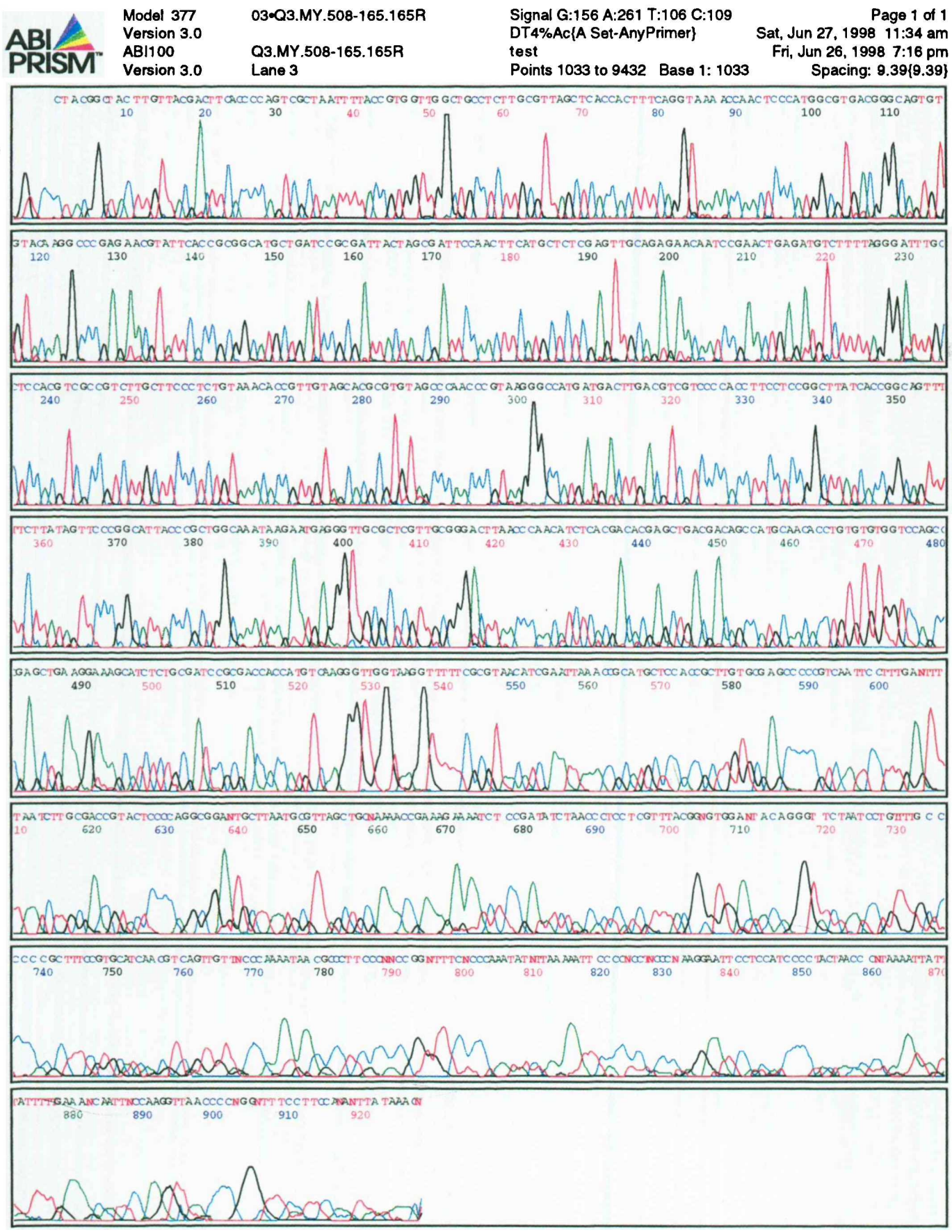


Fig. 5.13. Printed electropherogram of high throughput DNA sequencing provided by ABI PRISM ® 377 automated cycle sequencer. Approximately the first 500 bases are called with high confidence, extra bases are called with less certainty.

Other strategies adopted to overcome potential difficulties included:

Sequencing the 1450 base pair long 16S gene in 3 fragments of approximately 450-500 bases of high confidence base calling using primers at 500 base intervals; extra bases are called with less certainty, although each run gives up to 1000 bases of sequence data (Fig. 5.13). Because the sequence data is not an absolute in all cases, both DNA strands were also sequenced.

Complete length of 1450 base pair long bacterial 16S rDNA macromolecule from *L. bostrychophila* reproductive tissues were sequenced.

The sample sequences were compared with the available database of bacterial 16S r-RNA, which showed that the *L. bostrychophila* bacterial isolates belong to the α-subdivision of the Proteobacteria, where they are most closely related to the rickettsias.

Multiple aligned sequences of these rickettsial endosymbionts from 8 different populations of *L. bostrychophila* are presented in Table 5.2.

The phylogenetic relationships based on their sequence similarities between these insect endosymbionts were shown in Figure 5.14. This dendogram shows that the *L. bostrychophila* populations are infected with very closely related strains of rickettsiae.

These endosymbionts are grouped in 4 distinct, but very closely related sub-groups with equal distances according to their nucleotide sequences. Population 06 and 21 stand as single outliers (Group I and II). Two sister groups endosymbionts were isolated from population 08, 19 (Group III) and four populations of 05, 10; 23, 11 (Group IV).

There was 97.6% similarity in the 16S nucleotide sequence between bacteria from the liposcelid isolates and those living intracellularly in many other insects. These intracellular rickettsiae closely align with the sequence data which include the interoocytic *Wolbachia*. *Wolbachia* is the principal genus infecting insects, and causes a variety of reproduction alteration syndromes including parthenogenesis and cytoplasmic incompatibility.

```
        251                                                        300
pop 11  CCGACGATCT GTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGGACTGA
pop 23  CCGACGATCT GTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGGACTGA
pop 05  CCGACGATCT GTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGGACTGA
pop 10  CCGACGATCT GTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGGACTGA
pop 19  CCGACGATCT GTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGGACTGA
pop 08  CCGACGATCT GTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGGACTGA
pop 21  CCGACGATCT GTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGGACTGA
pop 06  CCGACGATCT GTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGGACTGA

        301                                                        350
pop 11  GACACGGCCC ACACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT
pop 23  GACACGGCCC ACACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT
pop 05  GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT
pop 10  GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT
pop 19  GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT
pop 08  GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT
pop 21  GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT
pop 06  GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT

        351                                                        400
pop 11  GGGCGAAAGC CTGATCCAGC AATACCGGGT GAGTGATGAA GGCCCTAGGG
pop 23  GGGCGAAAGC CTGATCCAGC AATACCGGGT GAGTGATGAA GGCCCTAGGG
pop 05  GGGCGAAAGC CTGATCCAGC AATACCGAGT GAGTGATGAA GGGCCTAGGG
pop 10  GGGCGAAAGC CTGATCCAGC AATACCGAGT GAGTGATGAA GGGCCTAGGG
pop 19  GGGCGAAAGC CTGATCCAGC AATACCGAGT GAGTGATAAA GGCCCTAGGG
pop 08  GGGCGAAAGC CTGATCCAGC AATACCGAGT GAGTGATAAA GGCCCTAGGG
pop 21  GGGCGAAAGC CTGATCCAGC AATACCGAGT GAGTGATAAA GGCCCTAGGG
pop 06  GGGCGAAAGC CTGATCCAGC AATACCGAGT GAGTGATAAA GGCCCTAGGG

        401                                                        450
pop 11  TTGTAAAGCT CTTTTAGCAA NGAAGATAAT GACGTTTCTT GCAGAAAAAG
pop 23  TTGTAAAGCT CTTTTAGCAA NGAAGATAAT GACGTTTCTT GCAGAAAAAG
pop 05  TTGTGAANCT CTTTTAGCAA NGAAGATAAT GACGTTACTT GCAGAAAAAG
pop 10  TTGTGAANCT CTTTTAGCAA NGAAGATAAT GACGTTACTT GCAGAAAAAG
pop 19  TTGTAAAGCT CTTTTAGCAA GGAAGATAAT GACGTTACTT GCAGAAAAAG
pop 08  TTGTAAAGCT CTTTTAGCAA GGAAGATAAT GACGTTACTT GCAGAAAAAG
pop 21  TTGTAAAGCT CTTTTAGCAA GGAAGATAAT GACGTTACTT GCAGAAAAAG
pop 06  TTGTAAAGCT CTTTTAGCAA GGAAGATAAT GACGTTACTT GCAGAAAAAG

        451                                                        500
pop 11  CCCCGGCTAA CTCCGTGCCA CCAGCCGCGG TAAGACGGAA GGGGCTAGCG
pop 23  CCCCGGCTAA CTCCGTGCCA CCAGCCGCGG TAAGACGGAA GGGGCTAGCG
pop 05  CCCCGGCTAA CTCCGTGCCA CCAGCCGCGG TAAGACGGAA GGGGCTAGCG
pop 10  CCCCGGCTAA CTCCGTGCCA CCAGCCGCGG TAAGACGGAA GGGGCTAGCG
pop 19  CCCCGGCTAA CTCCGTGCCA GCAGCCGCGG TAAGACGGAG GGGGCTAGCG
pop 08  CCCCGGCTAA CTCCGTGCCA GCAGCCGCGG TAAGACGGAG GGGGCTAGCG
pop 21  CCCCGGCTAA CTCCGTGCCA GCAGCCGCGG TAAGACGGAG GGGGCTAGCG
pop 06  CCCCGGCTAA CTCCGTGCCA GCAGCCGCGG TAAGACGGAG GGGGCTTGCT
```

```
        1                                                          50
pop 11  AGAGTTTGAT CCTGGCTCAA AACGAACGCT ATCGGTATGC TTAACACATG
pop 23  AGAGTTTGAT CCTGGCTCAA AACGAACGCT ATCGGTATGC TTAACACATG
pop 05  AGAGTTTGAT CCTGGCTCAA AACGAACGCT ATCGGTATGC TTAACACATG
pop 10  AGAGTTTGAT CCTGGCTCAA AACGAACGCT ATCGGTATGC TTAACACATG
pop 19  AGAGTTTGAT CCTGGCTCAA AACGAACGCT ATCGGTATGC TTAACACATG
pop 08  AGAGTTTGAT CCTGGCTCAA AACGAACGCT ATCGGTATGC TTAACACATG
pop 21  AGAGTTTGAT CCTGGCTCAA AACGAACGCT ATCGGTATGC TTAACACATG
pop 06  AGAGTTTGAT CCTGGCTCAA AACGAACGCT ATCGGTATGC TTAACACATG

        51                                                         100
pop 11  CAAGTCGGAC GGACTAATTA GGGCTTGCTC CAATTAGTTA GTGGCAGACG
pop 23  CAAGTCGGAC GGACTAATTA GGGCTTGCTC CAATTAGTTA GTGGCAGACG
pop 05  CAAGTCGGAC GGACTAATTA GGGCTTGCTC CAATTAGTTA GTGGCAGACG
pop 10  CAAGTCGGAC GGACTAATTA GGGCTTGCTC CAATTAGTTA GTGGCAGACG
pop 19  CAAGTCGGAC GGACTAATTA GGGCTTGCTC CAATTAGTTA GTGGCAGACG
pop 08  CAAGTCGGAC GGACTAATTA GGGCTTGCTC CAATTAGTTA GTGGCAGACG
pop 21  CAAGTCGGAC GGACTAATTA GGGCTTGCTC CAATTAGTTA GTGGCAGACG
pop 06  CAAGTCGGAC GGACTAATTA GGGCTTGCTC CAATTAGTTA GTGGCAGACG

        101                                                        150
pop 11  GGTGAGTNAC ACGTGGGAAT CTGCCCATCA GTACGGAATA ACTTTTAGAA
pop 23  GGTGAGTNAC ACGTGGGAAT CTGCCCATCA GTACGGAATA ACTTTTAGAA
pop 05  GGTGAGTAAC ACGTGGGAAT CTGCCCATCA GTACGGAATA ACTTTTAGAA
pop 10  GGTGAGTAAC ACGTGGGAAT CTGCCCATCA GTACGGAATA ACTTTTAGAA
pop 19  GGTGAGTAAC ACGTGGGAAT CTGCCCATCA GTACGGAATA ACTTTTAGAA
pop 08  GGTGAGTAAC ACGTGGGAAT CTGCCCATCA GTACGGAATA ACTTTTAGAA
pop 21  GGTGAGTAAC ACGTGGGAAT CTGCCCATCA GTACGGAATA ACTTTTAGAA
pop 06  GGTGAGTAAC ACGTGGGAAT CTGCCCATCA GTACGGAATA ACTTTTAGAA

        151                                                        200
pop 11  ATAAAAGCTA ATACCGTATA TTCTCTACAG AGGAAAGATT TATCGCTGAT
pop 23  ATAAAAGCTA ATACCGTATA TTCTCTACAG AGGAAAGATT TATCGCTGAT
pop 05  ATAAAAGCTA ATACCGTATA TTCTCTACAG AGGAAAGATT TATCGCTGAT
pop 10  ATAAAAGCTA ATACCGTATA TTCTCTACAG AGGAAAGATT TATCGCTGAT
pop 19  ATAAAAGCTA ATACCGTATA TTCTCTACAG AGGAAAGATT TATCGCTGAT
pop 08  ATAAAAGCTA ATACCGTATA TTCTCTACAG AGGAAAGATT TATCGCTGAT
pop 21  ATAAAAGCTA ATACCGTATA TTCTCTACAG AGGAAAGATT TATCGCTGAT
pop 06  ATAAAAGCTA ATACCGTATA TTCTCTACAG AGGAAAGATT TATCGCTGAT

        201                                                        250
pop 11  GGATGAGCCC GCGTCAGATT AGGTAGTTGG TGAGGTGACG GCTCACCAAG
pop 23  GGATGAGCCC GCGTCAGATT AGGTAGTTGG TGAGGTGACG GCTCACCAAG
pop 05  GGATGAGCCC GCGTCAGATT AGGTAGTTGG TGAGGTAACG GCTCACCAAG
pop 10  GGATGAGCCC GCGTCAGATT AGGTAGTTGG TGAGGTAACG GCTCACCAAG
pop 19  GGATGAGCCC GCGTCAGATT AGGTNGTTGG TGAGGTAACG GCTCACCAAG
pop 08  GGATGAGCCC GCGTCAGATT AGGTNGTTGG TGAGGTAACG GCTCACCAAG
pop 21  GGATGAGCCC GCGTCAGATT AGGTNGTTGG TGAGGTAACG GCTCACCAAG
pop 06  GGATGAGCCC GCGTCAGATT AGGTNGTTGG TGAGGTAACG GCTCACCAAG
```

Table 5.2. Multiple aligned sequences of bacterial 16S rDNA isolated from 8 populations of *L. bostrychophila.*

```
          751                                                       800
pop 11    GTAGTCCACG CCGTAAACGA TGAGTGCTAG ATATCGGAAG ATTCTCTTTC
pop 23    GTAGTCCACG CCGTAAACGA TGAGTGCTAG ATATCGGAAG ATTCTCTTTC
pop 05    GTAGTCCACG CCGTAAACGA TGAGTGCTAG ATATCGGAAG ATTCTCTTTC
pop 10    GTAGTCCACG CCGTAAACGA TGAGTGCTAG ATATCGGAAG ATTCTCTTTC
pop' 19   GTAGTCCACG CCGTAAACGA TGAGTGCTAG ATATCGGAAG ATTCTCTTTC
pop 08    GTAGTCCACG CCGTAAACGA TGAGTGCTAG ATATCGGAAG ATTCTCTTTC
pop 21    GTAGTCCACG CCGTAAACGA TGAGTGCTAG ATATCGGAAG ATTCTCTTTC
pop 06    GTAGTCCACG CCCTAAACGA TGAGTGCTAG ATATCGGAAG ATTCTCTTTC

          801                                                       850
pop 11    GGTTTCGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT ACGGTCGCAA
pop 23    GGTTTCGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT ACGGTCGCAA
pop 05    GGTTTCGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT ACGGTCGCAA
pop 10    GGTTTCGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT ACGGTCGCAA
pop 19    GGTTTCGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT ACGGTCGCAA
pop 08    GGTTTCGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT ACGGTCGCAA
pop 21    GGTTTCGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT ACGGTCGCAA
pop 06    GGTTTCGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT ACGGTCGCCA

          851                                                       900
pop 11    GATTAAAACT CAAAGGAATT GACGGGGGCT CGCACAAGCG GTGGAGCATG
pop 23    GATTAAAACT CAAAGGAATT GACGGGGGCT CGCACAAGCG GTGGAGCATG
pop 05    GATTAAAACT CAAAGGAATT GACGGGGGCT CGCACAAGCG GTGGAGCATG
pop 10    GATTAAAACT CAAAGGAATT GACGGGGGCT CGCACAAGCG GTGGAGCATG
pop 19    GATTAAAACT CAAAGGAATT GACGGGGGCT CGCACAAGCG GTGGAGCATG
pop 08    GATTAAAACT CAAAGGAATT GACGGGGGCT CGCACAAGCG GTGGAGCATG
pop 21    GATTAAAACT CAAAGGAATT GACGGGGGCT CGCACAAGCG GTGGAGCATG
pop 06    GATTAAAACT CCAAGGAATT GACAGGGGCT CGCACAAGCG GTGGAGCATG

          901                                                       950
pop 11    CGGTTTAATT CGATGTTACG CGAAAAACCT TACCAACCCT TGACATGGTG
pop 23    CGGTTTAATT CGATGTTACG CGAAAAACCT TACCAACCCT TGACATGGTG
pop 05    CGGTTTAATT CGATGTTACG CGAAAAACCT TACCAACCCT TGACATGGTG
pop 10    CGGTTTAATT CGATGTTACG CGAAAAACCT TACCAACCCT TGACATGGTG
pop 19    CGGTTTAATT CGATGTTACG CGAAAAACCT TACCAACCCT TGACATGGTG
pop 08    CGGTTTAATT CGATGTTACG CGAAAAACCT TACCAACCCT TGACATGGTG
pop 21    CGGTTTAATT CGATGTTACG CGAAAAACCT TACCAACCCT TGACATGGTG
pop 06    CGGTTTAATT CGATGTTTCG CGAAAAACCT TACCAACCCT TGACATGGTG

          951                                                      1000
pop 11    GTCGCGGATC GCAGAGATGC TTTCCTTCAG CTCGGCTGGA CCACACATAG
pop 23    GTCGCGGATC GCAGAGATGC TTTCCTTCAG CTCGGCTGGA CCACACATAG
pop 05    GTCGCGGATC GCAGAGATGC TTTCCTTCAG CTCGGCTGGA CCACACATAG
pop 10    GTCGCGGATC GCAGAGATGC TTTCCTTCAG CTCGGCTGGA CCACACATAG
pop 19    GTCGCGGATC GCAGAGATGC TTTCCTTCAG CTCGGCTGGA CCACACATAG
pop 08    GTCGCGGATC GCAGAGATGC TTTCCTTCAG CTCGGCTGGA CCACACATAG
pop 21    GTCGCGGATC GCAGAGATGC TTTCCTTCAG CTCGGCTGGA CCACACACAT
pop 06    GTCGCGGATC GCAGAGATGC TTTCCTTCAG CTCGGCTGGA CCACACATAG
```

```
          501                                                       550
pop 11    TTGTTTCGGA TTACTGGGCG TAAAGA.TGC GTAGGT.GGT TTAGTAAGTT
pop 23    TTGTTTCGGA TTACTGGGCG TAAAGA.TGC GTAGGT.GGT TTAGTAAGTT
pop 05    TTGTTCGGAA TTACTGGGCG TAAAGAGTGC GTAGGT.GGT TTAGTAAGTT
pop 10    TTGTTCGGAA TTACTGGGCG TAAAGAGTGC GTAGGT.GGT TTAGTAAGTT
pop 19    TTGTTCGGAA TTACTGGGCG TAAAGANTGC GTNGGC.GGT TTAGTAAGTT
pop 08    TTGTTCGGAA TTACTGGGCG TAAAGANTGC GTNGGC.GGT TTAGTAAGTT
pop 21    TTGTTCGGAA TTACTGGGCG TAAAGANTGC GTNGGC.GGT TTAGTAAGTT
pop 06    TTGTTCGGAA TTA.TGGGCG TAAAGA.TGC GTAGGT.GGT TTAGTAAGTT

          551                                                       600
pop 11    GGAAGTGAAA GCCCGGGGCT TAACCTCGGA ATTGCTTTCA AAACTACTAA
pop 23    GGAAGTGAAA GCCCGGGGCT TAACCTCGGA ATTGCTTTCA AAACTACTAA
pop 05    GGAAGTGAAA GCCCGGGGCT TAACCTCGGA ATTGCTTTCA AAACTACTAA
pop 10    GGAAGTGAAA GCCCGGGGCT TAACCTCGGA ATTGCTTTCA AAACTACTAA
pop 19    GGAAGTGAAA GCCCGGGGCT TAACCTCGGA ATTGCTTTCA AAACTACTAA
pop 08    GGAAGTGAAA GCCCGGGGCT TAACCTCGGA ATTGCTTTCA AAACTACTAA
pop 21    GGAAGTGAAA GCCCGGGGCT TAACCTCGGA ATTGCTTTCA AAACTACTAA
pop 06    GGAAGTGAAA GCCCGGGGCT TAACCTCGGA ATTGCTTTCA AAACTACTAA

          601                                                       650
pop 11    TCTAGAGTGT AGTAGGGGAT GATGGAATTC CTAGTGTAGA GGTGAAATTC
pop 23    TCTAGAGTGT AGTAGGGGAT GATGGAATTC CTAGTGTAGA GGTGAAATTC
pop 05    TCTAGAGTGT AGTAGGGGAT GATGGAATTC CTAGTGTAGA GGTGAAATTC
pop 10    TCTAGAGTGT AGTAGGGGAT GATGGAATTC CTAGTGTAGA GGTGAAATTC
pop 19    TCTAGAGTGT AGTAGGGGAT GATGGAATTC CTAGTGTAGA GGTGAAATTC
pop 08    TCTAGAGTGT AGTAGGGGAT GATGGAATTC CTAGTGTAGA GGTGAAATTC
pop 21    TCTAGAGTGT AGTAGGGGAT GATGGAATTC CTAGTGTAGA GGTGAAATTC
pop 06    TCTAGANTGT AGTAGGGGAT GATGGAATTC CTAGTGTAGA GGTGAAATTC

          651                                                       700
pop 11    TTAGATATTA GGAGGAACAC CGGTGGCGAA CGCGGTCATC TGGGCTACAA
pop 23    TTAGATATTA GGAGGAACAC CGGTGGCGAA CGCGGTCATC TGGGCTACAA
pop 05    TTAGATATTA GGAGGAACAC CGGTGGCGAA GGCGGTCATC TGGGCTACAA
pop 10    TTAGATATTA GGAGGAACAC CGGTGGCGAA GGCGGTCATC TGGGCTACAA
pop 19    TTAGATATTA GGAGGAACAC CGGTGGCGAA GGCGGTCAGC TGGGCTACAA
pop 08    TTAGATATTA GGAGGAACAC CGGTGGCGAA GGCGGTCAGC TGGGCTACAA
pop 21    TTAGATATTA GGAGGAACAC CGGTGGCGAA GGCGGTCAGC TGGGCTACAA
pop 06    TTAGATATTA GGAGGAACAC CNGTGGCGAA NGCGGTCATC TGGGCTACAA

          701                                                       750
pop' 11   CTGACGCTGA TGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
pop 23    CTGACGCTGA TGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
pop 05    CTGACGCTGA TGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
pop 10    CTGACGCTGA TGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
pop 19    CTGACGCTGA TGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
pop 08    CTGACGCTGA TGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
pop 21    CTGACGCTGA TGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
pop 06    CTGACGCTGA TGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
```

```
                                                     1050
        1001
pop 11  GTGTCGCATG GCTGTCGTCA GCTCGT.... ...GTCATGA GATGTTGGGT
pop 23  GTGTCGCATG GCTGTCGTCA GCTCGT.... ...GTCATGA GATGTTGGGT
pop 05  GTGTCGCATG GCTGTCGTCA GCTCGT.... ...GTCGTGA GATGTTGGGT
pop 10  GTGTCGCATG GCTGTCGTCA GCTCGT.... ...GTCATGA GATGTTGGGT
pop 19  GTGTCGCATG GCTGTCGTCA GCTCGT.... ...GTCCTGA GATGTTGGGT
pop 08  GTGTCGCATG GCTGTCGTCA GCTCGT.... ...GTCGTGA GATGTTGGGT
pop 21  GTGTGTCTTG GCATGGCTCT CCTCGT.... ...GTCCTGA GATGTTGGGT
pop 06  GTGTCGCATG GCTGTCGTCA GCTCGT.... ...GTCGTGA GATGTTGGGT

        1051                                         1100
pop 11  TAAGTCCCGC AACGAGCGCA ACCCTCATTC TTATTTGCCA GCGGGTAATG
pop 23  TAAGTCCCGC AACGAGCGCA ACCCTCATTC TTATTTGCCA GCGGGTAATG
pop 05  TAAGTCCCGC AACGAGCGCA ACCCTCATTC TTATTTGCCA GCGGGTAATG
pop 10  TAAGTCCCGC AACGAGCGCA ACCCTCATTC TTATTTGCCA GCGGGTAATG
pop 19  TAAGTCCCGC AACGAGCGCA ACCCTCATTC TTATTTGCCA GCGGGTAATG
pop 08  TAAGTCCCGC AACNAGCGCA ACCCTCATTC TTATTTGCCA GCGGGTAATG
pop 21  TAAGTCCCGC AACGAGCGCA ACCCTCATTC TTATTTGCCA GCGGGTTATG
pop 06  TAAGTCCCGC AACGAGCGCA ACCCTCATTC TTATTTGCCA GCGGGTAATG

        1101                                         1150
pop 11  CCGGGAACTA TAAGAAAACT GCCGGTGATA AGCCGGA.GG AAGGTGGGGA
pop 23  CCGGGAACTA TAAGAAAACT GCCGGTGATA AGCCGGA.GG AAGGTGGGGA
pop 05  CCGGGAACTA TAAGAAAACT GCCGGTGATA ACCCGGA.GG AAGGTGGGGA
pop 10  CCGGGAACTA TAAGAAAACT GCCGGTGATA AGCCGGA.GG AAGGTGGGGA
pop 19  CCGGGAACTA TAAGAAAACT GCCGGTGATA AGCCGGA.GG AAGGTGGGGA
pop 08  CCGGGAACTA TAAGAAAACT GCCGGTGATA AGCCGGA.GG AAGGTGGGGA
pop 21  CCGGGAACTA TAAGAAAACT GCCGGTGATA AGCCGGA.GG AAGGTGGGGA
pop 06  CCGGGAACTA TAAGAAAACT GCCGGTGATA AGCCGGA.GG AAGGTGGGGA

        1151                                         1200
pop 11  CAACTTCAAG TCATCATGGC CCTTACGGGT TGGGCTACAC CCGTGCTACA
pop 23  CAACTTCAAG TCATCATGGC CCTTACGGGT TGGGCTACAC GCGTGCTACA
pop 05  CAACTTCAAG TCATCATGGC CCTTACGGGT TGGGCTACAC GCGTGCTACA
pop 10  CAACTTCAAG TCATCATGGC CCTTACGGGT TGGGCTACAC CCGTGCTACA
pop 19  CAACTTCTAG TCATCATGGC CCTTACGGGT TGGGCTACAC CCGTGCTACA
pop 08  CAACTTCAAG TCATCATGGC CCTTACGGGT TGGGCTACAC CCGTGCTACA
pop 21  CAACTTCTAG TCCTCCTGGC CCTTACGGGT TGGGCTACAC CCGTGCTACA
pop 06  CAACTTCAAG TCATCATGGC CCTTACGGGT TGGGCTACAC CCGTGCTACA

        1201                                         1250
pop 11  ATGGTGTTTA CAGAGGGAAG CAAGACGGCG ACCTGGAGCA AATCCCTAAA
pop 23  ATGGTGTTTA CAGAGGGAAG CAAGACGGCG ACTTGGAGCA AATCCCTAAA
pop 05  ATGGTGTTTA CAGAGGGAAG CAAGACGGCG ACCTGGAGCA AATCCCTAAA
pop 10  ATGGTGTTTA CAGAGGGAAG CAAGACGGCG ACTTGGACCA AATCCCTAAA
pop 19  ATGGTGTTTA CAAACGGAAG CAAGACGGCA ACCTGGACCA AATCCCTAAA
pop 08  ACGGTGTTTA CAGAGGGAAG CAAGACGGCG ACCTGGAACA AATCCCTAAA
pop 21  ATGGTGTTTA CAGAGGGAAG CAAGACGGCG ACCTGGAGCA AATCCCTAAA
pop 06  ATGGTGTTTA CAGAGGGAAG CAAGACGGCG ACCTGGAGCA AATCCCTAAA

        1251                                         1300
pop 11  AGACATCTCA GTTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGTTGGA
pop 23  AGACATCTCA GTTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGTTGGA
pop 05  AGACATCTCA GTTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGTTGGA
pop 10  AGACATCTCA ATTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGTTGGA
pop 19  AGACATCTCA ATTCGGATTG TTCTCTGCAA CTCAAGAGCA TGAAGTTGGA
pop 08  AGACATCTCA GTTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGTTGGA
pop 21  AGACATCTCA ATTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGTTGGA
pop 06  AGACATCTCA GTTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGTTGGA

        1301                                         1350
pop 11  ATCCCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCTCGGGCC
pop 23  ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCTCGGGCC
pop 05  ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCTCGGGCC
pop 10  ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCTCGGGCC
pop 19  ATCGCTAGTA GTCGCGGATC AGCATGCCGC GGTGAATACG TTCTCGGGCC
pop 08  ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCTCGGGCC
pop 21  ATCGCTAGTA ATCCCGGATC AGCATGCCGC GGTGAATACG TTCTCGGGCC
pop 06  ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCTCGGGCC

        1351                                         1400
pop 11  TTGTACACAC TGCCCGTCAC GCCATGGGAG TTGGTTTTAC CTGAAGGTGG
pop 23  TTGTACACAC TGCCCGTCAC GCCATGGGAG TTGGTTTTAC CTGAAGGTGG
pop 05  TTGTACACAC TGCCCGTCAC GCCATGGGAG TTGGTTTTAC CTGAAGGTGG
pop 10  TTGTACACAC TGCCCGTCAC GCCATGGGAG TTGGTTTTAC CTGAAGGTGG
pop 19  TTGTACACAC TGCCCGTCAC GCCATGGGAG TTGGTTTTAC CTGAAGGTGG
pop 08  TTGTACACAC TGCCCCTCAC GCCATGGGAG TTGGTTTTAC CTGAAAGTGG
pop 21  TTGTACACAC TGCCCGTCAC GCCATGGGAG TTGGTTTTAC CTGAAGGTGG
pop 06  TTGTACACAC TGCCCGTCAC GCCATGGGAG TTGGTTTTAC CTGAAGGTGG

        1401                                         1450
pop 11  TGAGCTAACG CAAGACGCTG CCTACCCCCG GTAAATTAGC GACTGGGGTG
pop 23  TGAGCTAACG CAAGAAGCAG CCAACCACGG TAAAATTAGC GACTGGGGTG
pop 05  TGAGCTAACG CAAGAAGCAG CCAACCACGG TAAAATTAGC GACTGGGGTG
pop 10  TGAGCTAACG CAAGAGCCAC CCAACCACGG TAAAATTAGC GACTGGGGTG
pop 19  TGAGCTGACG CAAGAAGGCC CCGACCACCG TAAAATTAGC GACTGGGGTG
pop 08  TGAGCTAACG CCAGAAGCAG CCAACCACGG TAAAATTAGC GACTGGGGTG
pop 21  TGAGCTAACG CAAGAAGCAC CCAACCCCGG TAAAATTAGC GACTGGGTTA
pop 06  TGAGCTAACG CAAGAAGCAC CCAACCCCCG TAAAATTAGC GACTGGGGTG

        1451
pop 11  AA
pop 23  AA
pop 05  AA
pop 10  AA
pop 19  AA
pop 08  AA
pop 21  AA
pop 06  AA
```

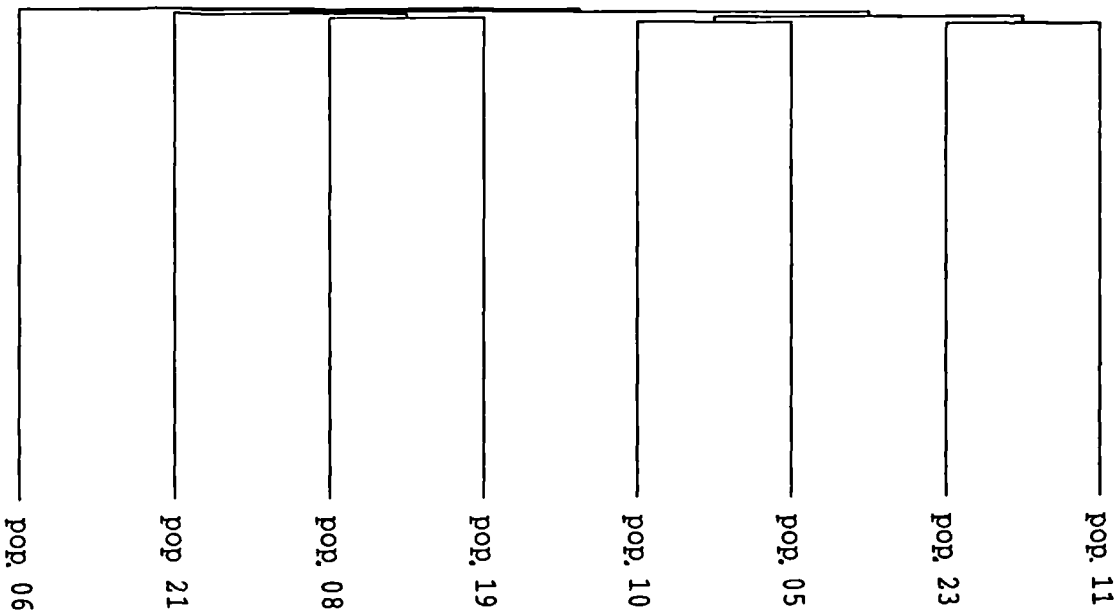


Fig. 5.14. Clustering relationship between 8 bacterial isolates from different strains of *L. bostrychophila* using pairwise alignment

5.3.4. Insect 12S rDNA Cloning results and sequence analysis

Genomic DNA extracted from fresh psocid tissues contained both insect and bacterial DNA. The insect DNA were selectively amplified using the 12SAI and 12BI, insect specific primers (O'Neill *et.al.*, 1992). These primers amplify insect mitochondrial 12S rRNA gene (encoding for rDNAs). Extraction, amplification, purification, cloning and other DNA handling techniques of this short subunit of psocid 12S were similar to that of the bacterial 16S rDNAs.

In comparison with the bacterial DNA, the PCR amplification of insect 12s rDNA was particularly difficult, although its cloning was relatively easy. Eleven of 15 *L. bostrychophila* populations gave satisfactory quantities of pure sequenceable DNA (Fig. 5. 15).

Fig. 5.15 shows the amplified insect 12S rDNAs from these 15 populations of *L. bostrychophila*. Two clones with 400bp fragments inserted were selected for each population (Fig. 5 16). Clones yielded highly concentrated DNA (0.2-2.5 mg/μl) for sequencing.

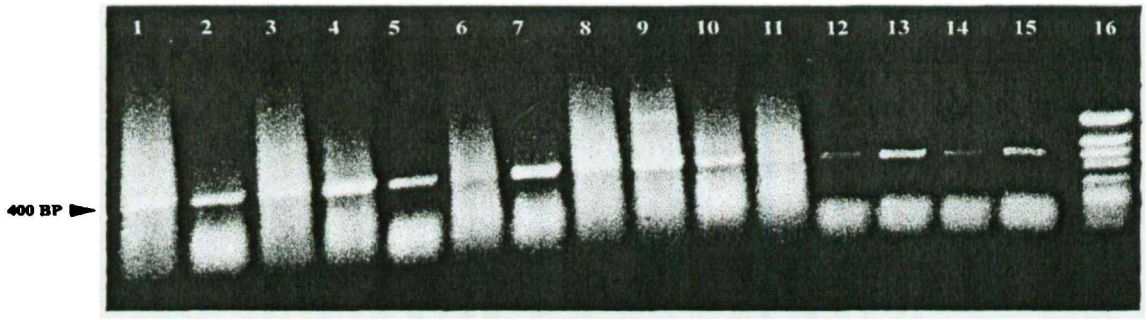


Fig. 5.15. Large scale PCR amplification of the 12S insect gene used for phylogenetic tree construction from 11 populations of *L. bostrychophila* (lane 1-15). The genomic DNA used for the amplification is DNAce clinipure extracted. This DNA is gel purified, phenol precipitated and 2μL of it ligated with pBluescript vector. Lane 16 is pBS SK /Hpa II marker.

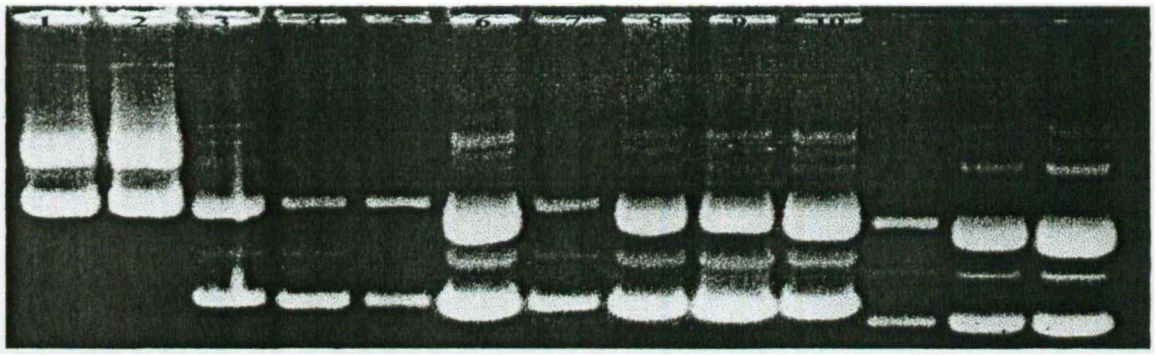


Fig. 5.16. Miniprep preparation of plasmid DNA carrying the insect 12S gene (Lane 1-10) to boost the sequenceable 12S rDNAs. This yielded a DNA concentration over 2500ng/λ. Lane 11-13 are standard pBluescript marker weighing 200, 1000, 2500ng/λ.

The complete sequences of 11 psocid populations are presented in (Table 5.3). Phylogenetic relationships of these insect strains based on their nucleotide sequence are shown in Figure 5.17.

```
          201                                                       250
12s. 09   TCTTTTCAGT CGGCGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 23   TCTTTTCAGT CGGCGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 05   TCTTTTCAGT CGGCGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 21   TCTTTTCAGT CGGCGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 06   TCTTTTCAGT CGGCGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 11   TCTTTTCAGT CGGCGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 26   TCTTTTCAGT CGGTGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 24   TCTTTTCAGT CGGTGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 08   TCCTTTCAGT CGGCGATATG GANGATGANA AAACTAAGTG TAGTAAGATT
12s. 13   TCTTTTCAGT CGGTGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT
12s. 19   TCTTTTCAGT CGGCGATATG GAGGATGAGA GAACTAAGTG TAGTAAGATT

          251                                                       300
12s. 09   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA AAAGGGGCCG
12s. 23   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA AAAGGGGCCG
12s. 05   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG
12s. 21   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG
12s. 06   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG
12s. 11   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG
12s. 26   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG
12s. 24   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG
12s. 08   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG
12s. 13   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG
12s. 19   GGGGTGGGTT ATCCATCAGG TCCATGGATC TCTAATTTGA GAAGGGGCCG

          301                                                       350
12s. 09   CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s. 23   CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s. 05   CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s. 21   CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s. 06   CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s. 11   CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s. 26   CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s. 24   CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s 08    CCACAAACCA TAGTTAATCT TTAAAAAAAA GTTATAAAAA TAATAGGGTA
12s 13    CCACAAACCA TAGTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA
12s. 19   CCACAAACCA TANTTAATCT TTAGAAAAAA GTTATAAGAA TAATAGGGTA

          351       365
12s. 09   TCTAATCCTA GTTTA
12s. 23   TCTAATCCTA GTTTA
12s. 05   TCTAATCCTA GTTTA
12s. 21   TCTAATCCTA GTTTA
12s. 06   TCTAATCCTA GTTTA
12s. 11   TCTAATCCTA GTTTA
12s. 26   TCTAATCCTA GTTTA
12s. 24   TCTAATCCTA GTTTA
12s. 08   TCTAATCCTA GTTTA
12s. 13   TCTAATCCTA GTTTA
12s. 19   TCTAATCCTA GTTTA
```

```
          1                                                         50
12s. 09   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 23   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 05   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 21   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 06   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 11   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 26   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 24   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 08   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 13   AAGAGCGACG GGCGATGTGT ACACTCCTTT TTACAATATT CAAGCTATTT
12s. 19   AAGAACAAAA GGCGATGTGT ACACTCCTTT TTTCTTTTTT CAAGCTATTT

          51                                                        100
12s. 09   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 23   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 05   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 21   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 06   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 11   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 26   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 24   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 08   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 13   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT
12s. 19   TAATAAAATA ACCTTACTTT TAAATTCATT TATTTTGTAT ATCAACAAAT

          101                                                       150
12s. 09   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 23   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 05   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 21   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 06   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 11   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 26   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 24   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 08   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 13   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT
12s. 19   TGATTCATCA AGATTTTTGA TCCACAAACT TTCAATACCA TAATCTTCAT

          151                                                       200
12s. 09   CTTTACCTGA CTTAACAGTT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 23   CTTTACCTGA CTTAACAGTT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 05   CTTTACCTGA CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 21   CTTTACCTGA CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 06   CTTTACCTGA CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 11   CTTTACCTGA CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 26   CTTTACCTGA CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 24   CTTTACCTGA CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 08   CTTTACCTGG CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGCTTT
12s. 13   CTTTACCTGA CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGC.TT
12s. 19   CTTTACCTGA CTTAACAGCT TAAAACTGAC AAAATAAAAA TTTATGCTTT
```

Table 5.3. Multiple aligned insect 12S rDNA sequences of 11 populations of *L. bostrychophila.*

The multiple aligning of the psocid DNA sequences shows that these 11 populations from different localities belong to one of three groups. Populations 13 and 19 represent the most dissimilar, while 08 is the third group. Population 08 in turn closely aligns with two sub-groups whereby population 09 and 23 have the most closely related sequences. Two sister groups were 09, 23 and 24, 26 ; 11, 06, 21, 05.

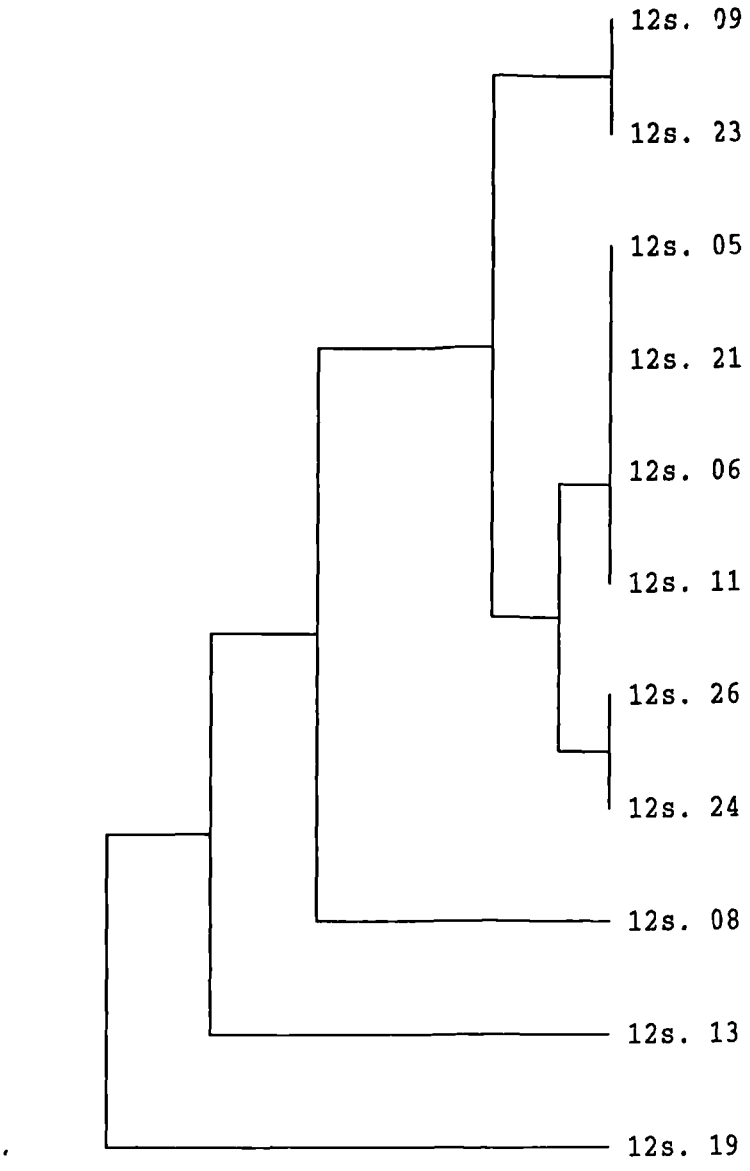


Fig. 5.17. Phylogenetic relationship of 11 populations of *L. bostrychophila* according to their 12S rDNA sequences.

5.3.5. Sequence variability in 16S and 12S genes.

Both 12S and 16S rDNA have shown some variation in nucleotide sequences, which in the case of the bacterial 16S gene can occur anywhere in the 1460 base pairs. Nucleotide differences were observed among these 8 bacterial isolates in 65 positions ignoring the ambiguities in the sequences from some isolates. However if the gene is subdivided into 14-15 units of 100 base pairs each, then the variability is less evenly spread with the last 750 being more variable region. The similarity score calculated using average similarity among all members of the 8 aligned sequences is shown in Figure 5.18. In this Figure the average similarity over the entire sequences is plotted at the middle position in the graph as a dotted line. The average similarity score of these 8 sequences is around 5.5. Given the degree of similarity among all sequences, the alignment procedure utilises previously aligned near relatives of the new sequences, the established secondary structural constraints, and sequence conservation pattern as guides.

Figure 5.19, shows the distribution of potential hot spots where the variations in the nucleotide sequences are likely to occur. This is calculated by giving value of 1 in all identical multiply aligned sequences, all other comparisons are given a value of 0.

As shown in figure 5.18, the first 300 base pairs are almost identical in all populations except two substitutions of A to G in the position of 297 and substitution of G to C in two bacterial isolates. More intense variations occur between 350 and 400, 500-600, 1000-1450. This arrangement in nucleotide variability in this gene makes the selective or partial sequencing of this gene uninformative. However the most dissimilar fragments of this gene are 350-550, around 700 and between 100-1450. Their similarity scores are 0.7, 0.8 and 0.3 respectively. Some incomplete, partial sequence database of the 16S can not therefore aligned with the complete data for phylogeny analysis.

The sequence divergence values among the 11 strains of *L. bostrychophila* shown in the small 12S gene is very limited (Figure 5.20). Nucleotide variations between different strains of *L. bostrychophila* were observed in total 13 positions. The number of substitutions ranged from 4 (1%) to 9 (2.25 %) in each strain of *L. bostrychophila.* These were concentrated in the position 1-10, 31-40, 151-160, 211-220, 231-240 and 321-330. The lowest similarity score for 12S was 0.9 in contrast to 0.3 in 16S.

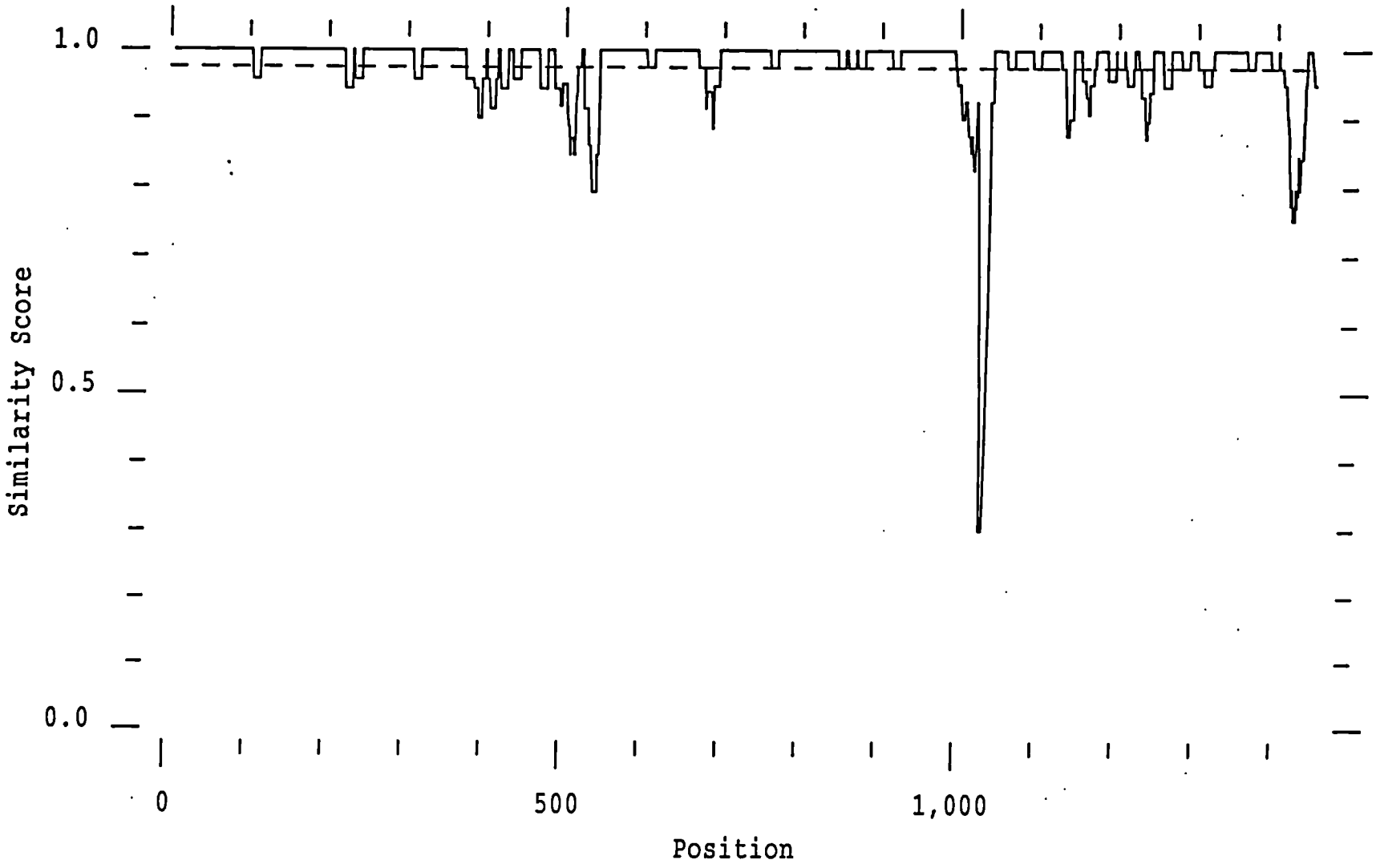


Fig. 5.18. Average similarity score of the multiply aligned 12S sequences from 8 populations of *L. bostrychophila*. the dotted line plotted in the middle position represent the average similarity.

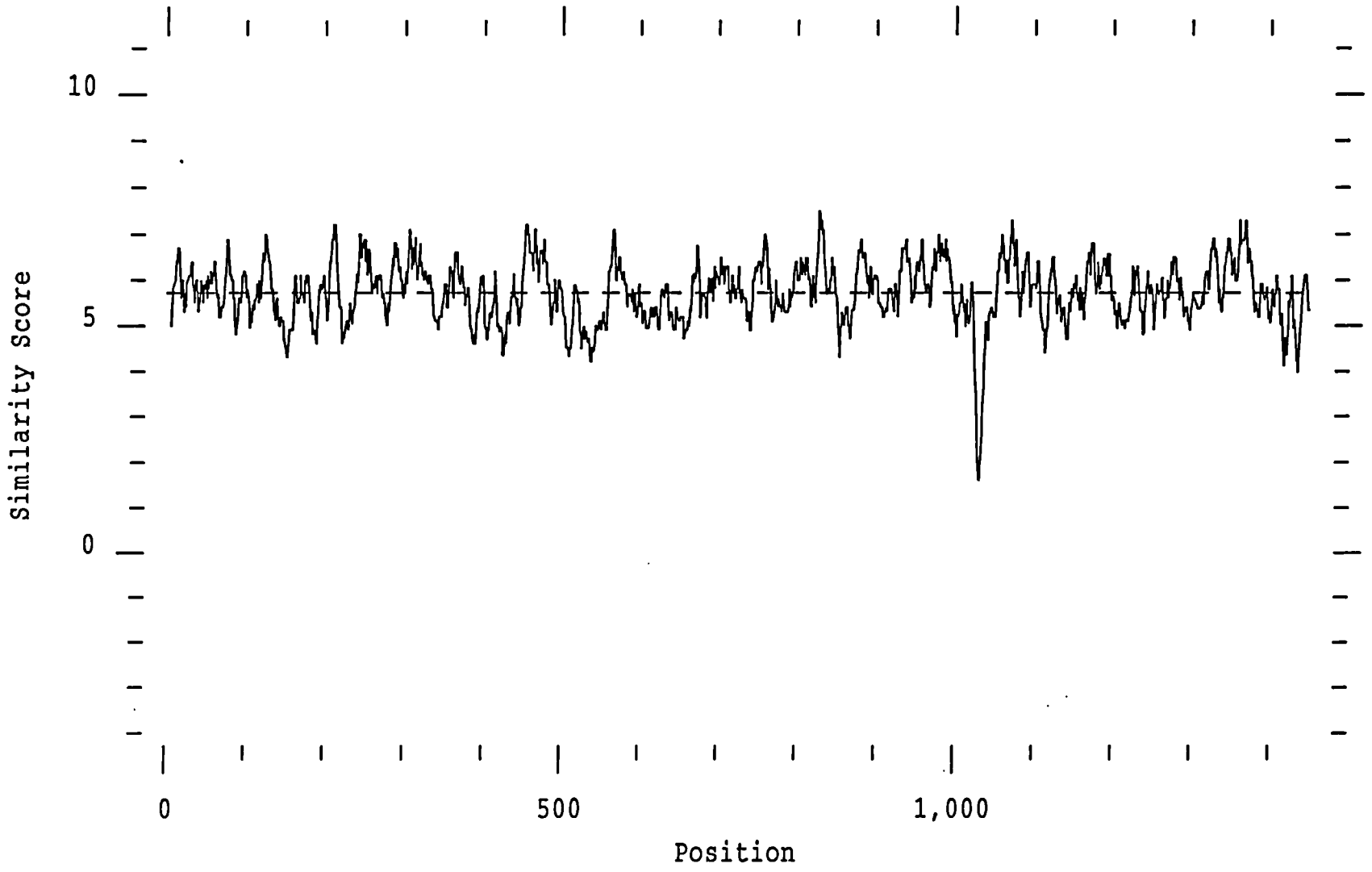


Fig. 5.19. Variation in nucleotide sequences throughout the 16S rDNA
bacterial gene. The dotted line plotted in the middle again represent the average similarity. The continuous line represent where all the multiply aligned sequences are identical and differences are shown where the straight line is disrupted.

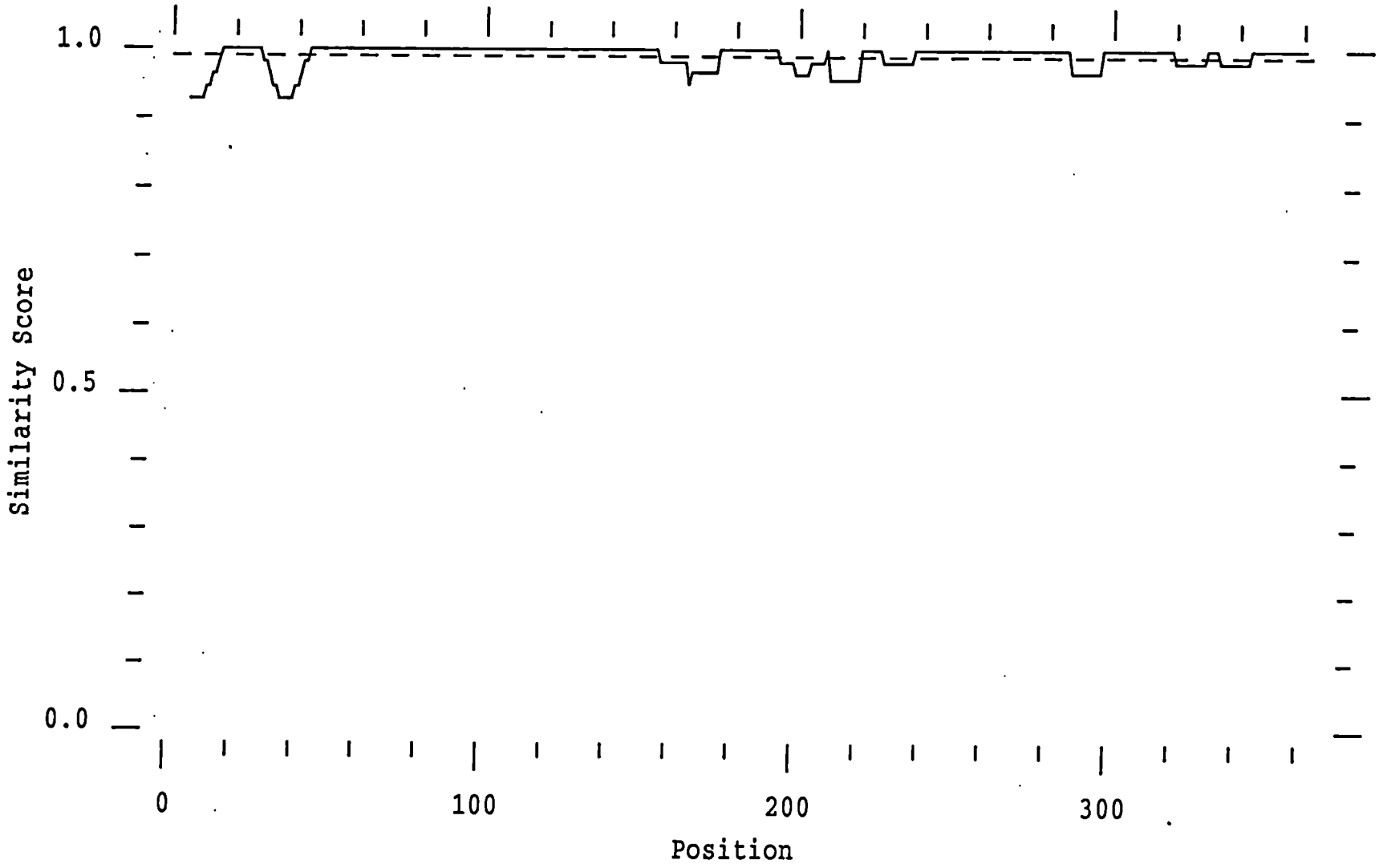


Fig. 5.20. Average similarity score in multiply aligned 12S insect gene for 11 populations of *L. bostrychophila*.

The majority of nucleotide variations (38%) occur by substituting A ↔ G and vice versa followed by A ↔ T (30%).

Simultaneous sequencing of insect 12S and bacterial 16S has not been achieved for all strains of *L .bostrychophila.* Strong concordance is shown in the clustering analysis between the 12S and 16S in some populations (Figures 5.14 and 5.17). As shown in these phylogenetic trees based on nucleotide divergence, some psocid strains that have clustered closely in their 12S gene, harboured a similar bacterial isolates indicating close parental or geographical link between separate populations of *L. bostrychophila.*

5.4. DISCUSSION

In this study, 30 populations of *L. bostrychophila* have been screened using gene sequencing. All these populations were invariably infected with the *Wolbachia*-like microrganisms. This screening process was also used to check the infection status of antibiotic treated individuals and two bisexual liposcelid species naturally lacking any infection. Both rifampicin treated and the bisexual psocids tested negative. The results from PCR are consistent with those from the EM investigations of the reproductive organs of these three psocid species.

Amplification of highly conserved bacterial 16S gene presents an opportunity to characterise the bacteria, placement and observe their biological variations.

Complete sequences of the 16S rDNA macromolecule of this bacterium from 8 populations have been successfully obtained and used to place the entomogenic *Rickettsia* in the α-Proteobacteria by using both generic and *Rickettsia* specific primers. This gene is also useful in the phylogenetic analysis of endosymbiotic bacteria by differentiating isolates that infect specific insect species (O'Neill *et al.*, 1992; Rousset *et.al.*, 1992; Stouthamer *et.al.*, 1993). This is particularly important in the association between endosymbionts and their psocid host. In *L. bostrychophila* parthenogenesis is obligatory and has resulted in a reduced within-population variability (Ali, 1994). With no outside genetic input, variability in the genome can only occur as a result of mutations. Hitherto the intimate association between insect and endosymbiont have evolved a variety of interactions that can promote mutualism (Fine, 1975). However, 16S rDNA evolves too slowly for detailed investigations of patterns and intertaxon transmission to be followed (Werren, 1997).

The 16S sequences determined from different strains of *L. bostrychophila* hosts have shown that all the populations are infected with very closely related rickettsial strains. The similarity score calculated using average similarity for all bacteria isolates was very high.

Since the rickettsiae are parasitic bacteria, typically found in an intracellular association with host tissues, the use of the 16S gene have facilitated their genetic characterisation. There are great differences among some of the genera in the family *Rickettsiaceae* (Weiss *et.al.*, 1984). Maintaining such a diverse genera as *Rickettsia* and *Coxiella* in the tribe *Rickettsieae* and *Ehrlichia* provides a useful guide in the isolation and identification of these microrganisms, but has no proven phylogenetic justifications (Weisburg *et.al.*, 1989; Ristic and Huxsoll, 1984).

Nevertheless the use of 16S gene for the taxonomic placement of bacteria is not definitive because those with identical 16S rDNA can be given different species designations (Fox *et. al.*, 1992). Therefore the results obtained using molecular tools have to be interpreted together with other data, generated using other methods.

Application of the biological species concept in bacterial endosymbionts in particular is problematic and in many cases the taxonomic issues of bacterial isolates both within (Clancy and Hoffmann, 1996) and between host species (Werren *et. al.*, 1995) are unresolved (Werren, 1997). There is also a tendency in the literature to call various bacteria isolates from different arthropods *Wolbachia pipientis* (intracellular endosymbionts from mosquito *Culex pipiens*) without a detailed characterisation (Werren, 1997).

Until this taxonomy issue is solved, the present intracellular bacteria found from psocid *L. bostrychophila* is designated as *Wolbachia*-like.

In this study the results obtained from molecular work are in accordance with that from histology and ultrastructure. Intracellular microrganisms isolated from *L. bostrychophila* are very similar to *Wolbachia* in ultrastructure, site of infection and polymorphism (Yusuf *et.al.*, 1999).

The second gene to be cloned and sequenced was the *L. bostrychophila* 12S rDNA short subunit, approximately 400 base pairs long. The amplification of this gene (12S) was useful as a control of the quality of the DNA extraction, because in some cases the PCR amplification of the bacterial 16S gene failed without any obvious explanation.

If 12S gene was successfully amplified in these cases other factors including low input of the target bacterial DNA, failure in the extraction technique or more importantly the presence of inhibitors in the template for amplification may be implicated. This was especially true when using the crude Proteinase-K based DNA extraction used by O'Neill *et.al.*, 1992. This was a rapid and inexpensive technique but was very insensitive and the silica based DNAce clinipure genomic DNA extraction technique using fresh animal tissues was favoured.

The insect 12S rDNA gene is universally distributed and useful in establishing phylogenetic relationships (Nigro and Grapputo, 1993). In this study the 12S rDNA is favoured over larger ribosomal genes like the 18S or 23S for the obvious advantages that 12S is shorter, can be amplified with universal primers and is extensively used as reference molecule because of its well documented role as a universal molecular clock (*Loc. cit.*). This gene gave satisfactory results in identifying closely related psocid strains. Genetic characterisation of 11 different populations of *L. bostrychophila* showed a limited variation of 1.25%. This is not unexpected since the level of variation between different psocid strains within one species is obviously less informative than analysing the relationships between groups.

The differences in sizes and scale of variation between the two rDNA genes makes the 12S useful for large scale taxonomic differentiation whilst the 16S gene is more useful to separate at the species level.

Simultaneous amplifications of insect host 12S and the 16S rDNA of infecting bacteria were useful in determining the level of concordance between the two.

Concordance in phylogeny between the two genes was shown in some populations where data on both genes were obtained. Closely related psocid strains with very similar 12S gene nucleotide sequences were infected with closely aligned bacterial isolates. This was particularly interesting as a similar study in the endosymbiont *Buchnera aphidicola,* have shown molecular concordance based on the 16S rDNA gene and their aphid host (Moran *et.al.*, 1993).

In other instances however no concordance was found between insect hosts and their endosymbionts (O'Neill *et.al.*, 1992; Rousset *et.al.*, 1992). This is thought to indicate that while a lineage of insects was possibly infected by symbionts only once, events leading to the infection have probably been more frequent and horizontal (intertaxon) in

nature. Therefore bacterial isolates that are identical or nearly identical in sequence are found in unrelated insect orders (Stouthamer *et.al.*, 1993).

Another important factor affecting the co-evolution between the microrganisms and their psocid host is the length of time of the association. Although divergence of different bacterial isolates based on their sequence variability can be estimated (Werren *et.al.*, 1995), determining when infections have occurred is more difficult. Other potential routes of infection include predatory, prey and other associated competitors (Werren, 1997).

Surveys using similar assays in other pest insect species have revealed the presence of similar endosymbionts from insect tissues (O'Neill *et.al.*, 1992). They concluded that the rickettsial distribution is much more pervasive than is currently recognised. 16S rDNA amplification from another parthenogenetic psocid species *Cerobasis guestfalica* also tested positive for *Wolbachia*, suggesting that these type of endosymbionts might actually be widespread in the parthenogenetic psocids.

CHAPTER SIX

EGG OUTPUT AND BACTERIAL DENSITY VARIATION AMONG L. *BOSTRYCHOPHILA* POPULATIONS

CHAPTER SIX

6.0. EGG OUTPUT AND BACTERIAL DENSITY VARIATION AMONG *L. BOSTRYCHOPHILA* POPULATIONS

6.1. INTRODUCTION

Variability has been demonstrated to exist between different populations and strains of *Liposcelis bostrychophila* (Ali, 1994). These variations manifest themselves in morphological, physiological or biochemical traits.

Populations of *Liposcelis bostrychophila* from different locations in the UK exhibit differences in the temporal pattern and quantity of eggs produced.

In chapter three it was shown that different individuals and populations of *L. bostrychophila* can carry varying loads of the intraoocytic bacterial endosymbionts. Possible relationships between the marked differences in egg output observed in *L. bostrychophila* and bacterial density are explored in this chapter.

The two temperature regimes used in the experiments throughout this chapter were 20 and 30 °C representing temperatures towards the lower and upper extremes between which eggs are laid. Egg production typically peaks within the first 3 weeks of adult life and tails off over a long period (Turner, 1994). The experimental period for these studies was limited to 7 weeks during which fecundity and mortality were recorded. These data were used to examine the range of fitness in populations of *L. bostrychophila.*

Factors affecting the bacterial growth in the insect cells are unknown. The relationship between bacterial abundance and insect fitness is very complex and reports in the literature are conflicting.

The associations between microbial symbionts and their multicellular host persist only if they increase at sufficiently similar rates that the microrganisms neither overgrow the association nor are diluted out by faster host growth (Wilkinson and Douglas, 1998). Virtually nothing is known about the regulatory processes operating at the level of the

host cell population, in most insect-microbe associations (Munson *et.al.*, 1991).

It is widely accepted that the persistence of the endosymbiosis is underpinned by an active co-ordination of host and symbiont growth, and that the rate of increase of the symbionts is regulated by the host (Douglas, 1994).

The only one notable case whereby the interactions between the insect host and its bacterial endosymbionts was investigated is that of aphid -*Buchnera* (Munson *et.al.*, 1991). In this symbiosis, the population of the bacterial cells is restricted to the cytoplasm of the bacteriocytes, located in the insect haemocoel (Baumann and Baumann, 1994; Humphreys and Douglas, 1997). The bacteria are transmitted vertically via the insect ovary and become incorporated with into the bacteriocytes of the embryo as these cells differentiate early in insect embryogenesis (Buchner, 1965; Hinde, 1971).

The relative importance of the host cell birth, growth and death to the regulation of the symbiosis varies with the developmental age of the insect. In *L. bostrychophila* the bacterial cells increase by both growth and division (Chapter 3). The chief mode of reduction of the bacterial endosymbionts is a lysis of the bacterial clusters during the mid to late reproductive period of a psocid's life.

The purpose of this part of the study addresses questions concerned with how the density of the bacteria changes with the psocid's age. Do different populations harbour different amounts of bacteria at the same stage in the reproductive cycle? Is the variation in egg output of *L. bostrychophila* populations related to their bacterial loads?

In this study the interaction between bacterial density and overall fitness and survivorship of different populations of *L. bostrychophila* is investigated.

6.2. MATERIAL AND METHODS

This study involves the thirty populations of *L. bostrychophila* obtained and reared as described in Chapter two (Section 2.2.1). Forty maturing teneral females (beginning to change to brown) were selected from each population and divided equally into two treatments, 20 and 30°C. Psocids were individually isolated in small vials. Food and oviposition substrate were provided by means of small pieces of food-coated filter

paper. Numbers of eggs laid and female mortality were recorded once a week for both temperatures. Additional teneral females from each population were assayed for the presence of the rickettsial cells. Both fluorescent and EM preparations were made.

The fluorescent staining of the ovaries using DAPI-like (4,6-diamidino-2-phenyl-indole) Flourochrome Hoechst 33258 was described in section 3.2.5 (Chapter 3).

Bacteria laden psocid tissues were recorded using 35 mm camera (Wild MPS 51S) mounted on an epiflourescent microscope (Dialux 20 EB Leitz).

6.3. RESULTS

6.3.1. Egg production at 20°C

At 20°C in most populations the females start egg production in the second week and reach maximum weekly fecundity in the 3rd-4th week (Fig. 6.. 1). The numbers of eggs laid per week then gradually reduce. In this cool environment close to the threshold temperature for egg production in *L. bostrychophila* reproduction continues for a long time span. Eggs were still being produced when experiment was terminated after seven weeks but the production was becoming very irregular and slow.

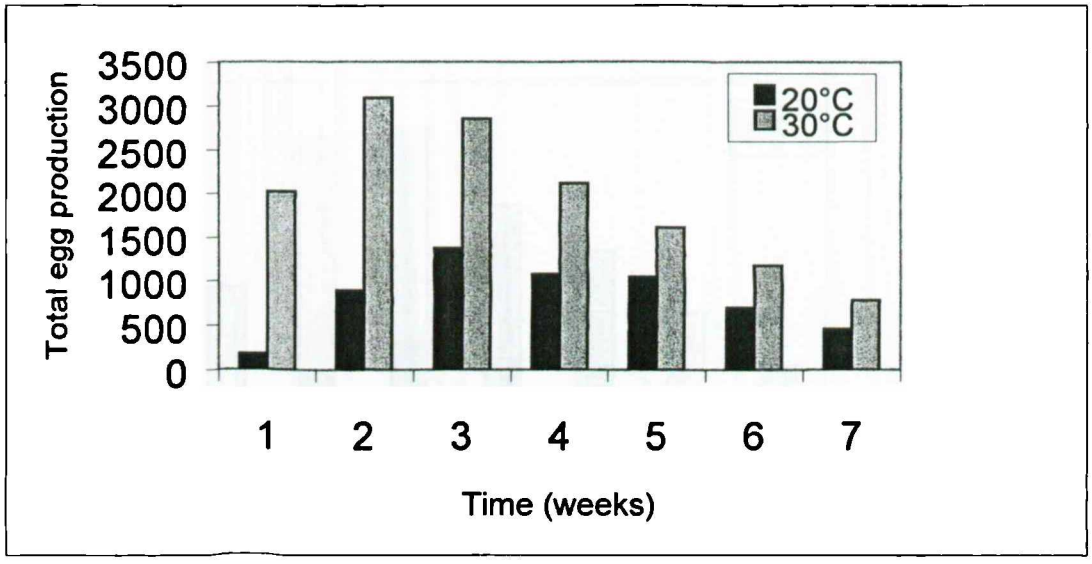


FIG. 6.1 Total egg production patterns of *L. bostrychophila* at 20 and 30°C in 7 weeks. *Cohort size (N) = 1200.*

The total number of eggs per week at 20°C was also consistently low in most populations. The mean egg output is lowest in the first week and reaches maximum of around 3-6 eggs per week per individual (*2SE = 0.85-2.51)* in week 3-4. The performance in terms of cumulative egg production for each population is shown in Fig 6.1-6.30. (population reproduction data are tabulated in appendix Table A6.1, population 1-30).

Analysis of variance on the total individual egg production per individual between the thirty populations of *L. bostrychophila* has shown significant differences between populations (at F=10.63, P = < 0.001). Populations 11, 12 13, 16, had the highest cumulative mean egg outputs, with population 16 reaching 27.02 *(± St Dev 1.83)* (Table A6.1; pop. 16). Populations 28, 23, 22 and 17 had the lowest mean cumulative egg output of around 5 *(± St Dev. 0.52-1)*.

6.3.2. Egg production at 30°C

At 30°C psocids generally have a higher rate of egg output per week. Mean egg production per individual in seven weeks at 30°C show markedly different patterns among the 30 populations. One way analysis of variance has also shown that the variation of egg output between populations is significant (F= 6.44, P = < 0.001).

The graphic representations of these data are shown in Figs. 6.1-5.31. Psocids kept at 30°C usually started production of eggs in week one and peak egg output was characteristically reached in the second week (Fig. 6. 1). Egg output remains high for maximum of two more weeks and then declines sharply. Table 6.1 shows that the populations 09, 11, 12, 13, 14, 22, 26 and 28 have produced largest number of eggs at 30°C. Egg output ranged from 2.75 *(2SE =1.83)* in week 7 to 9.21 *(± 2SE = 1.80)* in the second week of egg production in these relatively high egg output population.

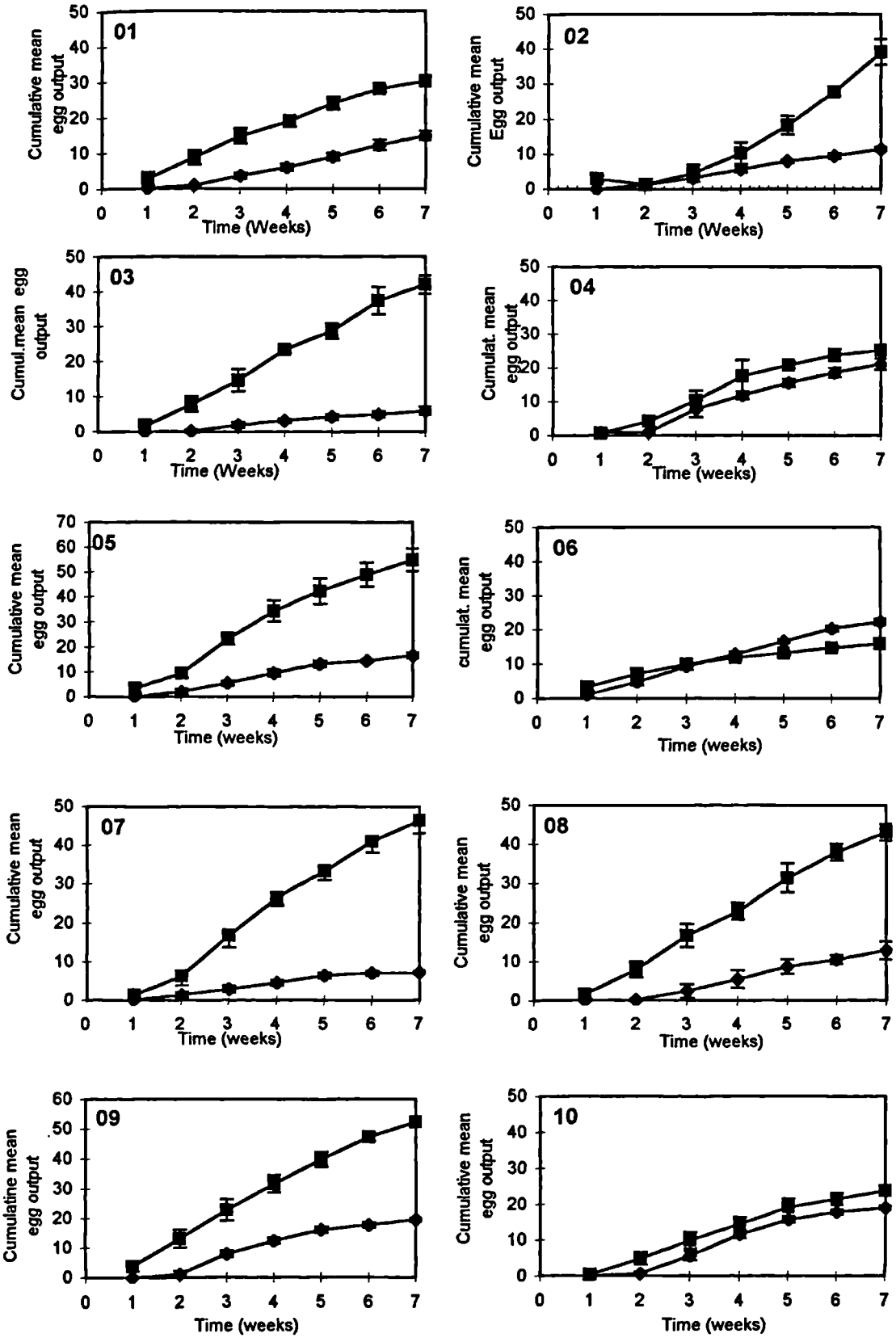
In the low egg output population the mean egg output per individual typically ranges from 3.50 eggs *(± 2SE = 1.91)* per individual in the 4-5th week of egg production to 1.75 *(± 2SE = 1.26)* in week 7 when experiments were terminated. The maximum mean egg output of most population was reached in the second week.

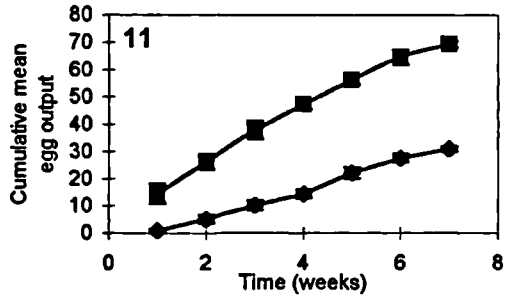
Cumulative mean figures shown in Figs. 6.2-6.3, in most populations are also consistently higher at 30°C than that of at 20°C. They range from 14 (Fig. A6.1 population 19) to 71 (population 13). In most populations the cumulative egg output remains around 50.

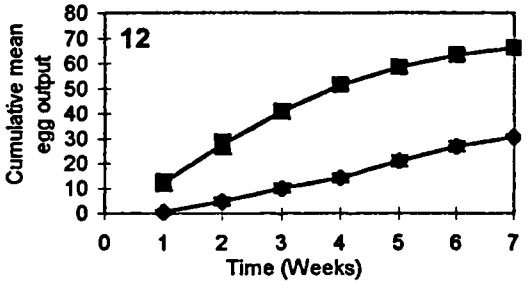
POP. No.	LOCATION	MORTALITY (%) AFTER 7 WEEKS AT 30°C	TOT. EGG PROD. 30°C AFTER 7 WEEKS	MORTALITY (%) AFTER 7 WEEKS AT 20°C	TOT. EGG PROD. 20°C AFTER 7 WEEKS	BACTERIA LOAD (No. colonies/0.1 mm^2)
01	Durham	70	371	35	208	43
02	Butley	80	485	65	113	69
03	Oxford	65	325	55	60	129
04	Seaton	70	278	30	377	31
05	Rotherham	80	401	25	296	27
06	Pontypool	80	172	60	316	19
07	Litchfield	65	415	75	74	22
08	Goole	70	409	70	103	98
09	Hull	55	592	30	319	28
10	Newcastle	75	195	20	345	30
11	Castleford	70	867	65	327	33
12	Cardiff	60	1040	70	345	78
13	Durham	75	860	35	426	40
14	Romford	75	624	55	320	29
15	Colchester	80	416	55	290	34
16	Sheffield	75	544	65	327	30
17	Hartlepool	65	487	40	82	25
18	Hertford	60	198	30	118	48
19	Yarmouth	80	85	35	114	47
20	Leeds	40	239	30	125	39
21	Croydon	70	439	60	202	31
22	Gosforth	75	548	35	97	77
23	Milford Haven	45	225	45	55	92
24	Southampton	50	403	30	196	55
25	Nottingham	35	283	20	120	70
26	Rotherham	65	656	65	123	52
27	Brighton	40	335	45	99	60
28	Newcastle	45	482	60	54	120
29	Kingston Upon Hull	60	275	40	84	92
30	Coventry	65	340	60	68	79

Table 6.1. Geographical distribution, egg production, mortality (%) and bacterial density of 30 populations of *L. bostrychophila*..

Fig. 6.2-6.3d (populations). Graphic representations of egg output of 30 populations of *L. bostrychophila* monitored over 7 weeks (± 2SE contains within the data set point symbols).
Diamonds (20°C), Squares (30°).




11
Cumulative mean egg output
Time (weeks)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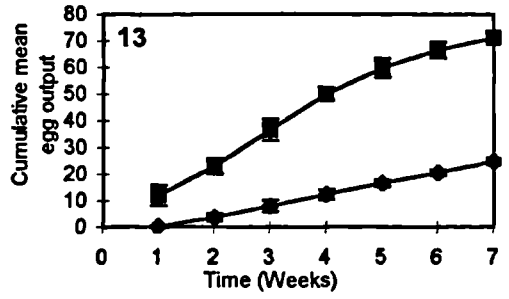
Cumulative mean egg output
Time (Weeks)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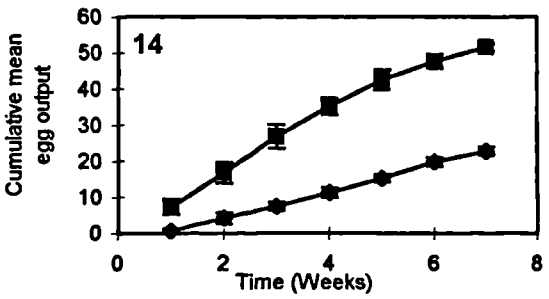
Cumulative mean egg output
Time (Weeks)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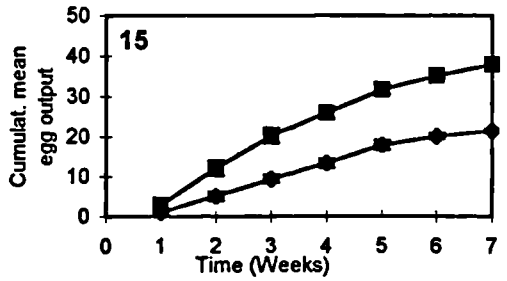
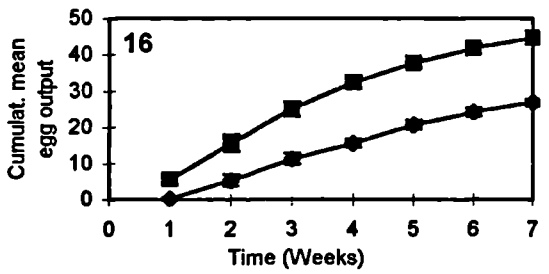
Cumulative mean egg output
Time (Weeks)


15
Cumulat. mean egg output
Time (Weeks)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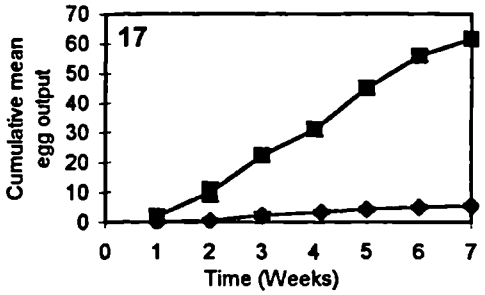
Cumulat. mean egg output
Time (Weeks)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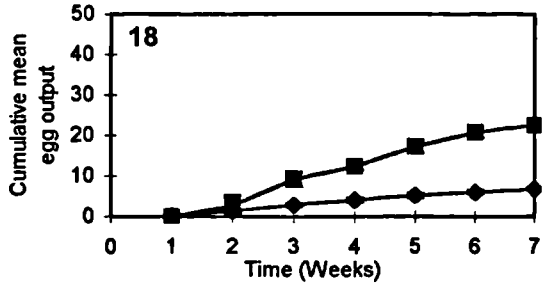
Cumulative mean egg output
Time (Weeks)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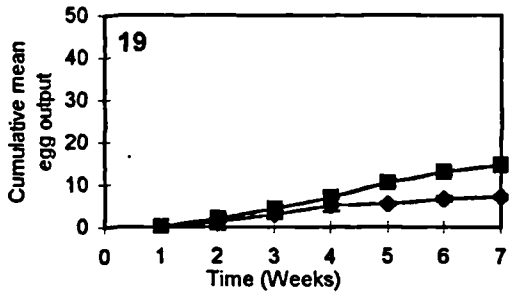
Cumulative mean egg output
Time (Weeks)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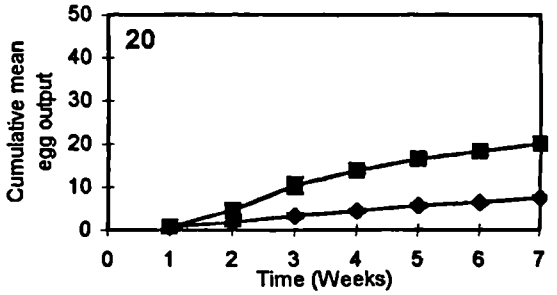
Cumulative mean egg output
Time (Weeks)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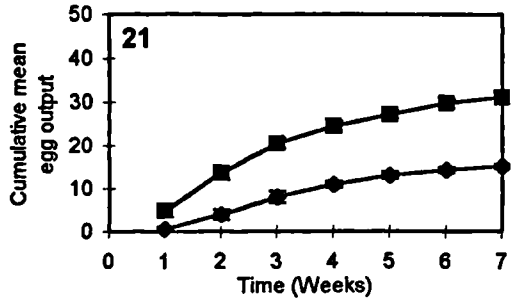
Cumulative mean egg output
Time (Weeks)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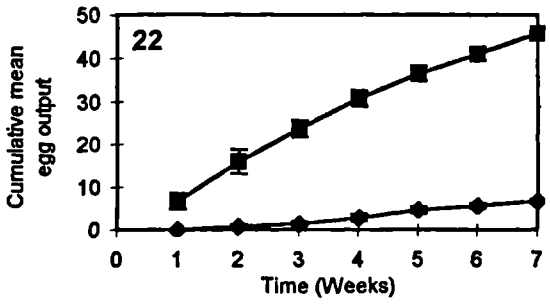
Cumulative mean egg output
Time (Weeks)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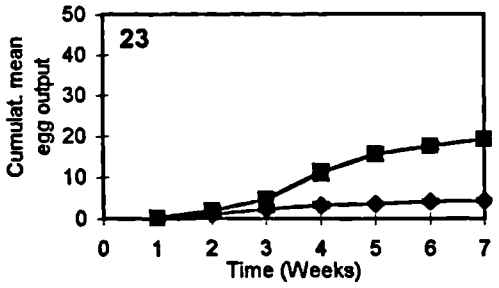
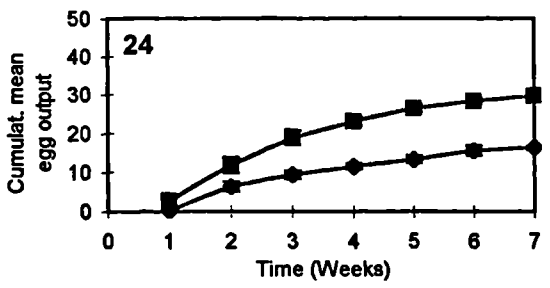
Cumulative mean egg output
Time (Weeks)


23
Cumulat. mean egg output
Time (Weeks)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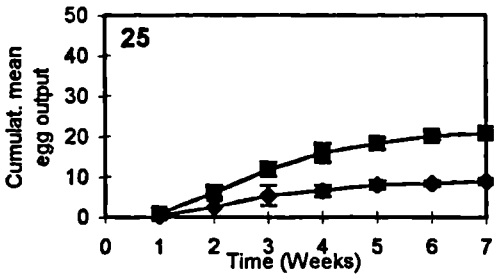
Cumulat. mean egg output
Time (Weeks)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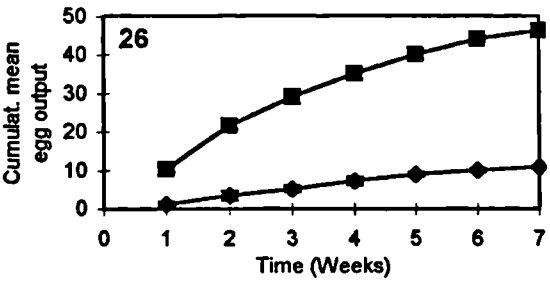
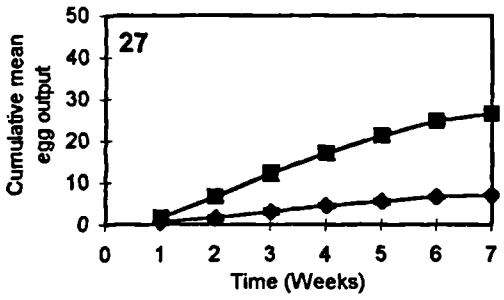
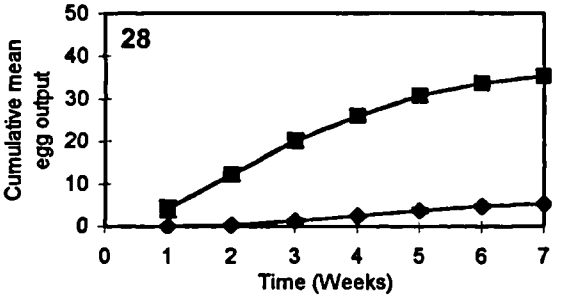
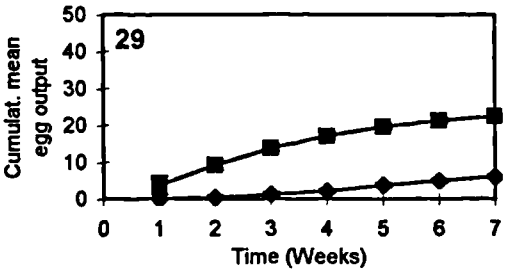
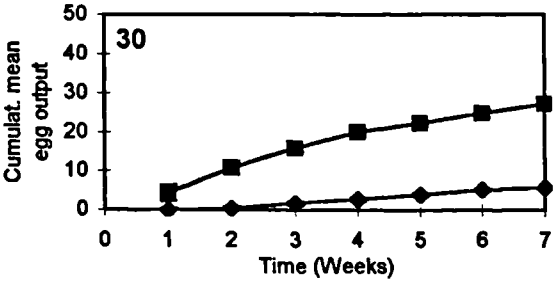
Cumulat. mean egg output
Time (Weeks)


26
Cumulat. mean egg output
Time (Weeks)


27
Cumulative mean egg output
Time (Weeks)


28
Cumulative mean egg output
Time (Weeks)


29
Cumulat. mean egg output
Time (Weeks)


30
Cumulat. mean egg output
Time (Weeks)

6.3.3. Comparison between 20°C and 30°C.

Although the majority of laboratory populations of *L. bostrychophila* produced more eggs at higher temperature, in some others the process was reversed. The total egg production in population reference number 04, 06, 10, 19 was higher at lower temperature of 20°C, while in some populations there were no appreciable differences in egg output between the two temperature regimes.

Fig. 6. 1, shows the overall egg production patterns per week of 30 populations of *L. bostrychophila* for both temperatures. At 20°C the egg production starts in the second weeks and peaks in the 3-4th week. At 30°C females start producing relatively large numbers of eggs in the first week and reach maximum production in the second to third week.

Cumulated mean egg production per individual in seven weeks at 20°C and 30°C show markedly different patterns among the 30 populations ranging from a maximum of 27 *(±1.96 St Dev 1.83)* (population 16) to 4.38 eggs *(± St Dev 0.67)* for population 23 at 20°C. At 30°C, the mean cumulative egg output per seven weeks reached a maximum of 102 eggs *(± 1.96 St Dev. = 23.3)* in population 02 and minimum of 14.71 eggs *(± 1.96 St Dev. = 1.26).*

6.3.4. Mortality rates at 20°C and 30°C

In most populations the higher temperature has a marked effect on egg production and also influences life span. High temperature maximised egg production and also hastened death in some populations (Fig. 6.32 and Fig. 6.33). The mortality rate at 20°C is usually lower or equal to that at 30°C.

In some cases at 30°C females produce a large number of eggs immediately before they die. At 20°C no such spurt was observed.

At 20°C about half (14 of 30) of the populations had mortality rates below 50% (Fig. 6.32), but at 30°C the mortality was reduced to 13% (4 of 30) (Fig. 6.33).

Mortality rates were typically between 60 to 80% at 30°C. The pattern of distribution of psocid mortality plotted against total egg production between the two temperature regimes is also different. At 20°C the egg production is lower and the rate of mortality widely distributed (Fig. 6.32). At 30°C egg production was relatively higher and the distribution in mortality rate was much smaller (Fig. 6.33). At both temperatures there was no correlation between egg production and mortality.

Fig. 6.32. Mortality rates of 30 populations *L. bostrychophila* held at 20°C for 7 weeks.

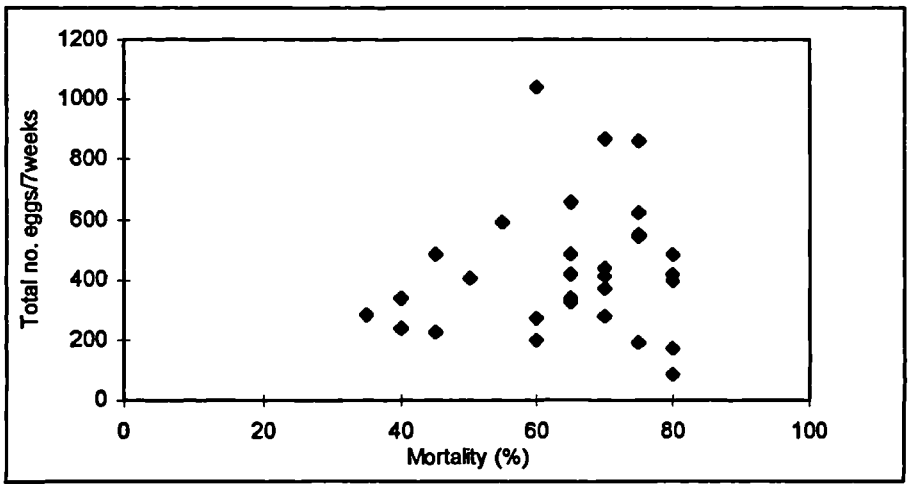


Fig. 6.33. Mortality rates of 30 populations *L. bostrychophila* held at 30°C for 7 weeks.

6.3.5. Bacterial Density

Data collected during this study have shown a wide variation in egg output among populations (Table 6.1). These differences were significant at both 20°C and 30°C ($F = 10.63$; $F = 6.447$ $P = < 0.001$).

Some egg production data of *L. bostrychophila* reported in the literature are also extremely variable (Ali, 1994). In chapter three, the differences in bacterial density between psocids of the same age and reared in same condition (temperature) were shown using EM. In this section the number of bacterial colonies were counted using fluorescent dye technology described in chapter four (Table 6.1.). Fluorescent staining showed that different individuals of *L. bostrychophila* had visibly different numbers of bacteria. Plate 4.10 (chapter 4), shows Flourochrome Hoechst 33258 stained *L. bostrychophila* oocytes. These tissues are filled with large number of luminescent bacterial particles. Populations with low bacterial loads on the contrary show a much reduced level of DNA-positive bodies (Plate 3.2, chapter 3). These condensed DNA were verified as bacterial images that are present in ovaries on the basis that they were only present in the infected *L. bostrychophila;* they were not found in the reproductive organs of bisexual species *L. corrodens* and correlated with electron transmission observations and PCR assays discussed in earlier chapters. The number of bacterial colonies visible in the ovariole tissues ranged from 19 to over 130 punctate fluorescing bacterial particles per 0.1mm^2 area. Figure 6.34, shows the correlation between the bacterial density and the total number of eggs per seven weeks. The correlation is negative ($r_s = 0.54$, $p = 0.01$), , since the total egg production increases with decreasing bacterial density and vice versa (Figure 6.34).

Increased heat has a major impact on the reproductive process of *L. bostrychophila*, not only in increasing the metabolic rate but also affecting these reproductive endosymbionts. As the oocyte develops so the bacterial cells also increase in number within it until the oocyte cytoplasm appeared to be filled. This build up of the bacteria occurred at both temperatures but the rate of increase is markedly different. At 30°C the egg production starts to drop from the first week, whilst at 20°C it reaches a peak in the second to third week.

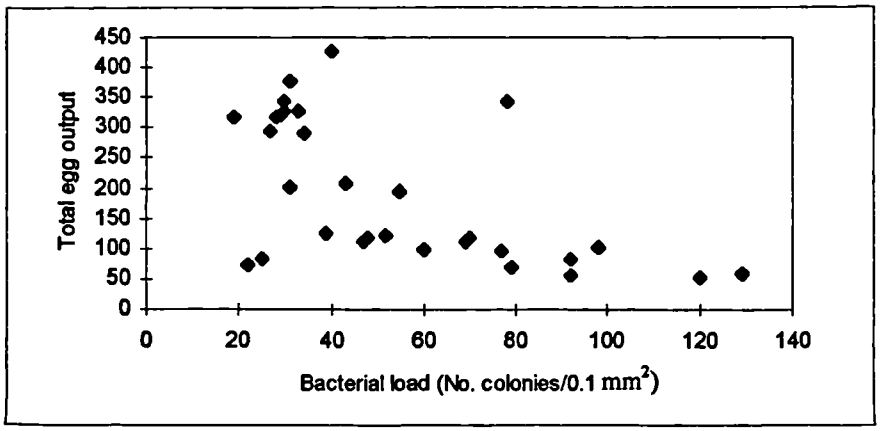


Fig. 6.34. Relationship between bacterial abundance and the total egg production in 30 populations of *L. bostrychophila*.

Bacterial density increased in the first 2^{nd}-3^{rd} weeks of egg production (Plate 3.9, Section 3.3.3.). The oocyte shown in this plate is from *L. bostrychophila* in the second week of egg production at 30°C. The cytoplasm of such an oocyte is occupied over a 1000 bacterial cells arranged in clusters. In some populations with high bacterial density, the oocyte starts to show some signs of degradation, eventually arresting egg production (Plate 3.9, Chapter 3).

6.3.6. Fitness and survivorship at the population level

Figure 6.35 shows the total egg production of the 30 populations at 20°C plotted against that for 30°C. In accordance with Q_{10} rule of van't Hoff and Arrhenius, it is expected that populations will approximately double their egg production when the temperature increases from 20°C to 30°C.

(Wigglesworth, 1965).

It was possible to classify the populations of *L. bostrychophila* in 3 categories based on their pattern of egg production at two temperatures.

- High temperature non-conformers. These populations perform better at 30°C by producing larger number of eggs than expected.
- Low temperature non-conformers. These populations are the opposite of the first group, more eggs were laid at low temperature and are suitably adapted to mild climate.

- Conformers. These populations have egg outputs at 30°C that are twice the production at 20°C.

There is broad scatter of the data points in Fig. 6.35 with some populations conforming the Q_{10} hypothesis, but others are non-conformers. Of particular interest are the 3 populations that produced 3-400 eggs at 20°C but only around 200 at 30°C.

These events can be seen more easily in Figs. 6.2-31 (e.g. population 4, 6, 10 and 19) which illustrates in graphic form the data of the fecundity and mortality tables for different *L. bostrychophila* populations.

Another factor affecting the fitness level of different populations of *L. bostrychophila* is the relationship between the psocid age and the bacterial load. Old females showed that an increased bacterial density and their entire oocytes were invaded by the bacteria causing some structural degeneration of the ovarial tissues of the insect host. At this stage the bacteria may reduce the level of insect fitness by arresting the egg production.

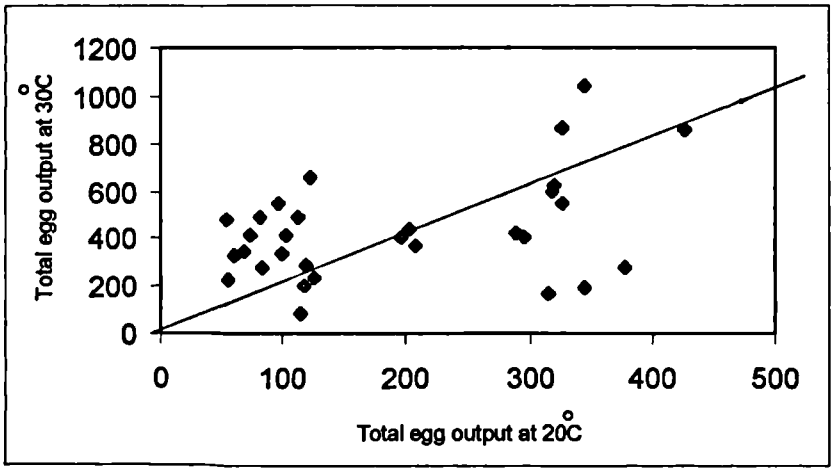


Fig. 6.35. Egg production at 20°C compared with that at 30°C for 30 populations of *L. bostrychophila. Cohort size (N=12000).*

6.3. DISCUSSION

Fecundity and mortality rates concerning 30 populations of *L. bostrychophila* collected from different locations in the UK have been calculated at 20°C and 30°C. Although *L. bostrychophila* is a tropical insect performing better at temperatures close to 30°C, some of the populations examined have shown physiological characteristics more suited to a temperate climate. Such populations are potentially more important in the UK as regards their pest status as they are less limited by the typical British temperatures (Turner, 1996).

Liposcelis bostrychophila populations from the present study have shown that the majority can survive and reproduce at low temperatures, whilst in some populations fecundity increased in low temperature regimes.

The influence of transovarially transmitted rickettsiae on host fertility of laboratory strains of *L. bostrychophila* was explored. In chapter four, the presence of sufficient numbers of the bacteria was shown to be necessary for reproduction. However the extremely high densities of bacteria found in the reproductive tissues of older females negatively correlates with egg production. The mechanisms involved are unknown.

Some similar studies have shown that the size of the insect influences the abundance of the endosymbionts in the insect host cells (Wilkinson and Douglas, 1998). Stevenson *et.al.*, (1995) also found that changes in both bacterial cell size and number contribute to differences in infected organ size in *Drosophila.* Ali (1994) found that variation in the body size in *L. bostrychophila* is related the geographical distribution. The relationship between this trait and the bacterial load was not investigated.

The key finding in this chapter is that the rate of egg output observed from different populations of *L. bostrychophila* and the load of the maternally inherited bacteria are correlated. Consistent with this conclusion is the evidence from other studies that the principal control over the bacterial load is exerted at different developmental stages of insect. The volume of bacterial cells present in the teneral females is determined during nymphal development, the cells increase in volume regularly through larval development (Douglas and Dixon, 1987), at a rate correlated with the overall growth

rate of the insects (Adams, 1996). Wilkinson and Douglas (1998) found that the number of bacteria in the maternal tissue of teneral aphids was significantly lower than in than in the basal embryo, suggesting a limited bacterial cell death during larval development. The implication is that the number of bacterial cells in teneral insects may be dictated primarily by the number that differentiate in the early embryo and the number of times that the bacterial cells divide during embryogenesis. In aphids the number is not fixed, for the clones of pea aphid studied by Douglas and Dixon (1987) counted 60 bacteriocytes, as did Hongoh and Ishikawa (1994), while in other instances 75-120 bacteriocytes were counted in other clones of same species (Rahbe *et.al.*, 1993; Wilkinson and Douglas (1998).

The number of bacterial cells may be also influenced by various developmental factors, including embryo size, gestation time and the rearing temperature. In *L. bostrychophila* the number of bacterial cells depends on the population, age of the psocid and the incubated temperature. The evidence in this study, that the control over number and volume of bacterial cells in the psocid oocytes was correlated is readily compatible with this view.

CHAPTER SEVEN
GENERAL DISCUSSION

CHAPTER SEVEN

7.0. GENERAL DISCUSSION

With few exceptions there seems to be little reported pathology attributable to the RLO. The only possible symptoms that could be defined as pathological are lysis of the cellular boundaries in tissues with high densities of the microrganisms and the amount of yolk material in the mature eggs of *Wolbachia*-laden individuals. Yolk serves principally as a source of nutrients during embryonic development (Counce, 1972). Low amounts of lipid and protein yolk in the eggs are found to be directly related in juvenile mortality in some insects (Mahowald, 1972). In both of the bisexual liposcelids examined, the egg yolk was the major component of the egg. In comparison the oocyte of *L. bostrychophila* has minimal amount of yolk material.

Despite the apparently major changes that they induce in developing oocytes, the rickettsiae found in *L. bostrychophila* appear to be relatively harmless parasites, as is the case with many other intracellular symbionts[1] of insects. There are distinct advantages to the host and the parasite in terms of enhanced reproductive and dispersal potential with induced parthenogenesis. The colonisation of new habitats by *L. bostrychophila* is however facilitated by the fact that food storage facilities, linked with man, are commonly available and present relatively constant habitat worldwide. So, there is a lesser need of the genetic variability potentially generated by sexual reproduction. Despite this, some degree of variability is known to exist among different populations of *L. bostrychophila* in terms of egg output as well as allozyme patterns (Ali, 1994).

In some other cases of *Rickettsia* infected insects, Wright and Barr (1980) also concluded that high densities of *Wolbachia pipientis* of young mosquitoes can be pathological as the result of a loss of some infected germ cells. Wright *et.al.*, (1978) found necrotic pupal nurse cells and Yen and Barr in 1974, described pathological cells in pupal testis, which they attributed to *Wolbachia* infection.

Beside these limited cases, the relationship between the insect host and it s intimately associated endosymbionts appears to be less damaging at least at low densities. But

[1] ***The term symbiont, as it is customarily employed in the extensive literature of the intracellular symbionts of insects, refers rather to the invariable association of insect and microrganisms and to the exquisite equilibrium which characterised it, rather than to proven mutualism (Hertig).***

they fall short of being harmless, as there is a variable degree of degeneration of host cells.

The developmental stages of an egg at which symbionts enter appear to vary with species (Koch, 1960). Symbionts make their appearance in different developmental stages in oogenesis. The humoral and /or immune responses of the insect to these endosymbiotic bacteria are poorly understood (Warr, 1981), but limited use of sites where endosymbionts are found suggests a level of specificity in the movements as well as the chemical processes governing symbiosis. Symbiotic microrganisms' movement is passive, and governed by migrations and engulfing reactions on the part of the host's ovarian cells (Brooks, 1962). The ability of some endosymbionts in the larvae to move from one part of the body, by being carried passively with the haemolymph to ovarian cells, has been reported (Brooks and Richards, 1955). In *L. bostrychophila* oocytes are already infected. The stimuli and the modes of movements by the bacteria which culminate in the infection of the oocytes is unknown. Having been transported to the ovaries, there still remains the question of how these immobile intracellular microrganisms pass through membranes and reach the oocyte cytoplasm and eventually the embryo.

High-resolution electron microscope investigations of the parthenogenetic psocid L. bostrychophila have shown that every oocyte is invaded by RLO. All other life stages of this psocid species were also found to be infected. The life cycles of the parasites and that of the insect host are thus tightly coupled. Similar ultrastructural investigations of the reproductive systems of two bisexual liposcelid species showed them be RLO-free.

L. bostrychophila is completely parthenogenetic and genetically isolated from other populations of the same species. Parthenogenesis i.e. not requiring males, even indirectly to fertilise eggs, in most insect species operates as fail-safe mechanism. In *L. bostrychophila* however, parthenogenesis is obligatory and has resulted in reduced population variability and the loss of any outside genetic input, heavily relying on mutation to evolve and adapt. To compensate for these negative aspects of asexual life, parthenonotes have a far greater intrinsic rate of increase and range than the bisexual species. *L. bostrychophila* for example is the world's most widely distributed psocid species.

The phylogenetic relationships between the parthenogenetic *L. bostrychophila* and its closely related bisexual liposcelids are largely unknown. The morphological differences between the members of the sub-group IID (*L. bostrychophila* sub-group) of the genus *Liposcelis,* which is made of an aggregate of parthenogenetic and bisexual species, are in some cases small. There is a need for more stringent criteria to be applied to test whether *L. bostrychophila* is a true species or a parthenogenetic form of the one of the bisexual species in the assemblage. Mockford (1971) observed similar trends in his study of parthenogenesis in the Psocoptera. He identified at least three thelytokous species namely *Echmepteryx hageni, Caecilius auranticus* and *Peripsocus quadrifasciatus* with bisexual forms in some part of their range. In two other species, having obligatory thelytokous forms, *Psocus bipunctatus* and *Trichadenotecnum alexandrae,* females are also capable of facultative parthenogenesis (Schneider, 1955; Mockford, 1971). It is not inconceivable that similar mechanisms are in operation in the case of *L. bostrychophila* and that it may be the parthenogenetic form of the bisexual species in the complex. For example *L. corrodens* is a bisexual species that is morphologically similar to *L. bostrychophila.*
The status of *L. bostrychophila* can be settled by conducting genetic characterisation and specific gene sequencing of the members of genus *Liposcelis.*

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APPENDIX

Pop. No	Location	County	Number of Psocids
01	Durham	Durham	5 Individuals
02	Butley	Suffolk	2 ,, ,,
03	Oxford	Oxfordshire	8 ,, ,,
04	Seaton	Northumberland	10 ,, ,,
05	Rotherham	South Yorkshire	25 ,, ,,
06	Pontypool	Gwent	10 ,, ,,
07	Litchfield	Hampshire	58 ,, ,,
08	Goole	Humberside	8 ,, ,,
09	Hull	Humberside	8 ,, ,,
10	Newcastle	Tyne & Wear	25 ,, ,,
11	Castleford	West Yorkshire	5 ,, ,,
12	Cardiff	South Glamorgan	5 ,, ,,
13	Durham	Durham	30 ,, ,,
14	Romford	Essex	55 ,, ,,
15	Colchester	Essex	3 ,, ,,
16	Sheffield	South Yorkshire	3 ,, ,,
17	Hartlepool	Cleveland	20 ,, ,,
18	Hertford	Hertfordshire	10 ,, ,,
19	Yarmouth	Isle of Wight	20 ,, ,,
20	Leeds	West Yorkshire	1 ,, ,,
21	Croydon	Greater London	6 ,, ,,
22	Gosforth	Tyne & Wear	15 ,, ,,
23	Milford Haven	Dyfed	6 ,, ,,
24	Southampton	Hampshire	4 ,, ,,
25	Nottingham	Nottinghamshire	1 ,, ,,
26	Rotherham	South Yorkshire	7 ,, ,,
27	Brighton	East Sussex	17 ,, ,,
28	Newcastle	Tyne & Wear	35 ,, ,,
29	Kingston Upon Hull	Humberside	1 ,, ,,
30	Coventry	West Midlands	1 ,, ,,

Table A2.1. Geographical distribution of 30 populations of *L. bostrychophila* used for experiments throughout this study.

Table A4.1. The egg production of population 05 of *L. bostrychophila* treated with 0.03% Tetracycline, Cohort size *N=20.*

Time (Weeks)	1	2	3	4	5	6	7	8	9	10	Cohort egg production
Total No. Eggs/Week	5.0	39.0	59.0	49.0	17.0	15.0	17.0	10.0	3.0	3.0	217.0
Mean No. Eggs/Ind./W	0.25	2.05	3.69	4.08	1.70	1.67	2.13	1.43	0.60	0.60	
Cumulative Mean	0.25	2.30	5.99	10.07	11.77	13.44	15.57	16.99	17.59	18.19	
2SE	0.24	0.74	0.96	1.73	0.66	0.72	0.61	0.64	0.41	0.66	
Cumulative Mortality (%)	0.00	5.0	20.0	40.0	40.0	45.0	50.0	55.0	65.0	65.0	65.0

Table A4.2 Controls. Population 05 of *L. bostrychophila* fed on only standard diet. Cohort size *N=20.*

Time (Weeks)	1	2	3	4	5	6	7	8	9	10	Cohort egg production
Total No. Eggs/Week	23.0	49.0	71.0	63.0	30.0	28.0	25.0	10.0	4.0	6.0	309.0
Mean No. Eggs/Ind./W	1.28	2.72	4.44	3.94	2.50	2.80	2.50	1.25	0.50	0.75	
Cumulative Mean	1.28	4.00	8.44	12.38	14.88	17.68	20.18	21.43	21.93	22.68	
2SE	069	0.65	0.86	0.83	0.95	1.05	0.98	0.96	0.52	0.61	
Cumulative Mortality (%)	10.0	10.0	20.0	20.0	40.0	50.0	50.0	60.0	60.0	60.0	60.0

Table A4.3. Egg production of population 01 of *L. bostrychophila* treated with 0.03% Tetracycline, Cohort size *N=20*

Time (Weeks)	1	2	3	4	5	6	7	8	9	10	Cohort egg Production
Total No. Eggs/Week	39.0	71.0	78.0	40.0	25.0	21.0	18.0	8.0	3.0	11.0	314.0
Mean No. Eggs/Ind./W	2.05	3.94	4.33	2.67	2.27	2.10	1.80	0.89	0.43	1.57	
Cumulative Mean	2.05	6.00	10.33	13.00	15.27	17.37	19.17	20.06	20.49	22.13	
2SE	0.73	0.92	1.53	1.01	1.18	0.62	1.02	0.76	0.37	0.79	
Cumulative Mortality(%)	5.00	10.0	10.0	25.0	45.0	50.0	50.0	55.0	60.0	60.0	60.0

Table A4.4 Controls. Population 01 of *L. bostrychophila* fed on only standard diet. Cohort size *N=20.*

Time (Weeks)	1	2	3	4	5	6	7	8	9	10	Cohort egg Production
Total No. Eggs/Week	101.0	115.0	88.0	67.0	21.0	25.0	18.0	8.0	4.0	6.0	453.0
Mean No. Eggs/Ind./W	5.32	6.39	5.18	4.47	1.91	2.50	2.25	1.00	0.50	0.75	
Cumulative Mean	5.32	11.71	16.89	21.36	23.27	25.77	28.02	29.02	29.52	30.27	
2SE	1.49	1.53	1.76	1.30	0.62	1.06	1.27	0.64	0.37	0.32	
Cumulative Mortality(%)	5.0	10.0	15.0	25.0	45.0	50.0	60.0	60.0	60.0	60.0	60.0

Table A4.5. The egg production of population 09 of *L. bostrychophila* treated with Heat at 37°C and no tetracycline (Controls), Cohort size *N=20*

Time (Weeks)	1	2	3	4	5	6	7	8	9	Cohort egg Production
Total No. Eggs/Week	138.0	117.0	63.0	67.0	33.0	28.0	9.0	3.0	0.00	458
Mean No. Eggs/Ind./W	6.90	7.31	4.20	5.15	2.75	2.55	1.00	0.50	0.00	
Cumulative Mean	6.90	14.21	18.41	23.57	26.32	28.86	29.86	30.36	30.36	
2SE	1.84	1.80	1.02	1.01	1.39	1.04	0.57	0.67	0.00	
Cumulative Mortality(%)	0.00	20.0	25.0	35.0	40.0	45.0	55.0	70.0	80.0	80.0

Table A4.6 Egg production of population 09 of *L. bostrychophila* treated with heat and 1% Tetracycline, Cohort size *N=20.*

Time (Weeks)	1	2	3	4	5	6	7	8	Cohort egg Production
Total No. Eggs/W	69. 0	44.0	91.0	63.0	18.0	6.0	2.0	0.00	293
Mean No. Eggs/Ind./W	3.45	2.93	7.00	7.00	2.25	0.86	0.40	0.00	
Cumulative Mean	3.45	6.38	13.38	20.38	22.63	23.49	23.89	23.89	
2SE	1.17	0.80	1.90	2.51	1.10	1.08	0.78	0.00	
Cumulative Mortality(%)	0.0	25.0	35.0	55.0	60.0	65.0	75.0	85.0	85.0

Table A4.7 The egg production of population 09 of *L. bostrychophila* treated with Heat at 37°C and 0.3% Tetracycline, Cohort size *N=20.*

Time (Weeks)	1	2	3	4	5	6	7	8	9	Cohort egg Production
Total No. Eggs/Week	37.0	67.0	88.0	133.0	43.0	18.0	28.0	15.0	0.00	429.0
Mean No. Eggs/Ind./ W	2.06	3.72	4.89	7.39	3.31	1.50	2.55	1.36	0.00	
Cumulative Mean	2.06	5.78	10.67	18.06	21.36	22.86	25.41	26.77	26.77	
2SE	0.77	1.24	1.22	2.69	1.62	0.85	3.09	1.80	0.00	
Cumulative Mortality(%)	10.0	10.0	10.0	10.0	35.0	40.0	45.0	45.0	45.0	45.0

Table A4.8. Egg production of population 09 of *L. bostrychophila* treated with Heat at 37°C and 0.03% Tetracycline. Cohort size *N=20.*

Time (Weeks)	1	2	3	4	5	Cohort egg Production
Total No. Eggs/Week	116.0	115.0	85.0	12.0	6.0	334.0
Mean No. Eggs/Ind./W	6.82	7.19	6.54	1.00	0.67	
Cumulative Mean	6.82	14.01	20.55	21.55	22.22	
2SE	1.90	1.99	1.44	0.67	0.75	
Cumulative Mortality(%)	15.0	20.0	30.0	30.0	40.0	40.0

RIFAMPICIN TREATMENTS

Table A4.9. Egg production of *L. bostrychophila* population of 05 treated with 1% rifampicin at 30°C.

Time (Weeks)	1	2	3	4	5	6	7	Cohort egg production
Tot. No. Eggs/Week	0.00	8.00	12.00	23.00	11.00	2.00	0.00	56
Mean No. Eggs/Ind./W	0.00	0.47	0.71	1.77	1.38	0.33	0.00	
Cumulative mean	0.00	0.47	1.18	2.95	4.33	4.66	4.66	
2SE	0.00	0.35	0.55	0.92	0.87	0.36	0.00	
Cumulative mortality (%)	10.00	15.00	15.00	25.00	45.00	50.00	50.00	50.00

Table A4.10. Juvenile development of *L. bostrychophila* treated with 1% rifampicin. All eggs laid by all females in the above table (1% treated) were monitored.

Number of eggs	Egg Mortality	Number of 1st instar larvae	Number nymphs reaching II instar (%)	Number nymphs reaching III instar (%)	Number nymphs reaching IV instar (%)	Teneral female	Juvenile mortality (%)
56	34(60.8%)	22 (39.2)	7 (31.8%)	4 (18.1%)	2 (9.0)	2 (9.0)	91.0

Table A4.11. Egg production of *L. bostrychophila* population of 05 fed on standard diet without the rifampicin at 30°C.

Time (Weeks)	1	2	3	4	5	6	7	Cohort egg production
Tot. No. Eggs/Week	86.00	111.0	67.00	126.00	95.00	39.00	24.00	548
Mean No. Eggs/Ind./W	4.53	6.94	5.15	9.69	8.64	3.90	3.43	
Cumulative mean	4.53	11.47	16.62	26.31	34.95	38.85	42.28	
2SE	0.83	1.36	0.87	1.64	1.47	1.04	0.56	
Cumulative mortality (%)	5.00	30.00	35.00	40.00	45.00	50.00	55.00	55.00

Table A4.12. Egg production of *L. bostrychophila* population of 05 treated combined 1% rifampicin and tetracycline at 30°C.

Time (Weeks)	1	2	3	4	5	6	7	Cohort egg production
Tot. No. Eggs/Week	9.00	14.00	9.00	6.00	3.00	4.00	2.00	47.00
Mean No. Eggs/Ind./W	0.47	1.00	0.82	0.60	0.33	0.50	0.33	
Cumulative mean	0.47	1.47	2.29	2.89	3.22	3.72	4.01	
2SE	0.42	0.62	0.64	0.47	0.31	0.33	0.23	
Cumulative mortality (%)	5.00	30.00	35.00	40.00	45.00	50.00	60.00	60.00

Table A4.13. Egg production of *L. bostrychophila* population of 05 fed on standard diet without the combination of rifampicin and tetracycline at 30°C.

Time (Weeks)	1	2	3	4	5	6	7	Cohort egg production
Tot. No. Eggs/Week	96.00	178.00	162.00	99.00	61.00	38.00	27.00	661
Mean No. Eggs/Ind./W	5.33	11.87	12.46	9.00	6.78	4.75	3.86	
Cumulative mean	5.33	17.20	29.66	38.66	45.44	50.19	54.05	
2SE	0.95	3.15	2.79	1.70	1.29	1.38	0.93	
Cumulative mortality (%)	5.00	20.00	30.00	40.00	50.00	55.00	60.00	60.00

Juvenile development

Table A4.14. Juvenile development of *L. bostrychophila* treated with 1% tetracycline and controls fed with standard diet. Mean duration nymphal stages (Days) ±2SE = 0.38. Tetracycline treated; C, Controls.

Number of 1st instar larvae		Duration nymphal stages (Days)		Nymphal mortality (%)		Nymphs reaching adult (%)		Number eggs laid Week I		Number eggs laid Week II		Number eggs laid Week III		Adult mortality (%)	
T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C
20	20	9	7	15	20	80	85	107	141	191	207	64	123	12.5	11

Table A4.15. Juvenile development of *L. bostrychophila* treated with 1% rifampicin and controls fed with standard diet. Mean duration nymphal stages (Days) ±2SE = 0.46. T, Tetracycline treated; C, Controls.

Number of 1st instar larvae		Duration nymphal stages (Days)		Nymphal; mortality (%)		Nymphs reaching adult (%)		Number eggs laid Week I		Number eggs laid Week II		Number eggs laid Week III		Adult mortality	
T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C
20	20	12	9	30	10	70	90	44	193	59	271	17	106	7	16.5

Table A4.16. Juvenile development of *L. corrodens* treated with 1% tetracycline, rifampicin and controls fed with standard diet.

	Tetracycline Treated	Rifampicin Treated	Controls
Number of 1st instar larvae	20	20	20
Duration nymphal stages (Days)	11	11	11
Nymphal mortality (%)	25	30	25
Nymphs reaching adult (%)	75	70	75
Number eggs laid Week I	16	21	13
Number eggs laid Week II	18	25	20
Number eggs laid Week III	14	18	15
Adult mortality (%)	23	14	15

Tables A6.1. Summarises the egg production and mortalities of the 30 *L. bostrychophila* populations (01-30) at 20 and 30°C.

Population 01. 20°C								
Total no. egg/week	2	12	40	3	41	47	31	208
Mean egg/ind	0.11	0.80	2.67	2.50	2.93	3.36	2.58	
Cumul. Mean	0.11	0.91	3.58	6.08	9.01	12.36	14.95	
Stdev	0.47	1.32	1.68	1.91	1.94	2.82	2.31	
2SE	0.22	0.16	0.85	1.00	1.02	1.48	1.31	Cohort Mort.
Mortality	2	2	0	1	0	0	2	7
Cumul.Mort.	10	20	20	25	25	25	35	35
30°C								
Total no. egg/week	53	114	73	45	41	32	13	371
Mean egg/ind	2.79	6.00	6.08	4.09	5.13	4.00	2.17	
Cumul. Mean	2.79	8.79	14.87	18.96	24.09	28.09	30.26	
Stdev	3.92	4.37	3.87	2.55	2.53	1.31	1.17	
2SE	1.76	1.96	2.19	1.51	1.75	0.91	0.87	
Mortality	1	0	7	1	3	0	2	14
Cumul.Mort.	5	5	40	45	65	65	70	70

Population 02. 20°C								
Total no. egg/week	3	14	21	25	23	13	14	113
Mean egg/ind	0.11	1.17	1.91	2.50	2.30	1.44	2.00	
Cumul. Mean	0.11	1.28	3.19	5.69	7.99	9.43	11.43	
Stdev	0.00	1.34	1.87	1.43	0.67	1.13	0.82	
2SE	0.00	0.76	1.10	0.89	0.42	0.74	0.60	
Mortality	3.00	5	1	1	0	1	2	13
Cumul.Mort.	15	40	45	50	50	55	65	65
30°C								
Total no. egg/week	101	223	242	229	251	238	250	1537
Mean egg/ind	6.77	14.91	16.14	15.31	16.79	15.87	16.69	
Cumul. Mean	3.00	21.68	37.82	53.12	69.92	85.79	102.47	
Stdev	13.43	29.34	21.10	18.35	20.75	21.03	23.31	
2SE	6.38	15.37	11.94	11.99	15.37	16.83	22.84	
Mortality	3	3	2	3	2	1	2	16
Cumul.Mort.	15	30	40	55	65	70	80	80

Population 03. 20°C								
Total no. egg/week	0	3	21	10	10		10	60
Mean egg/ind	0.00	0.25	1.75	1.11	1.11	0.67	1.11	
Cumul. Mean	0.00	0.25	2.00	3.11	4.22	4.89	6.00	
Stdev	0.00	0.62	1.48	0.93	1.27	1.32	1.76	
2SE	0.00	0.35	0.84	0.61	0.83	0.86	1.15	
Mortality	5	3	0	3	0	0	0	11
Cumul.Mort.	25	40	40	55	55	55	55	55
30°C								
Total no. egg/week	23	56	54	62	36	62	32	325
Mean egg/ind	1.64	6.22	6.75	8.86	5.14	8.86	4.57	
Cumul. Mean	1.64	7.87	14.62	23.47	28.62	37.47	42.04	
Stdev	3.41	3.23	4.53	1.35	2.97	5.30	3.51	
2SE	1.79	2.11	3.14	1.00	2.20	3.93	2.60	
Mortality	6	5	1	1	0	0	0	13
Cumul.Mort.	30	55	60	65	65	65	65	65

Population 04. 20°C								
Total no. egg/week	18	0	132	76	70	48	33	377
Mean egg/ind	0.95	0.00	6.95	4.00	3.68	3.00	2.54	
Cumul. Mean	0.95	0.95	7.89	11.89	15.58	18.58	21.12	
Stdev	1.81	0.00	5.59	2.58	2.54	2.80	3.20	
2SE	0.81	0.00	2.51	1.16	1.14	1.37	1.68	
Mortality	1	0	0	0	0	3	2.	6
Cumul.Mort.	5	5	5	5	5	20	30	30
30°C								
Total no. egg/week	11	48	75	86	29	21	8	278
Mean egg/ind	0.73	3.43	6.25	7.17	3.22	3.00	1.33	
Cumul. Mean	0.73	4.16	10.41	17.58	20.80	23.80	25.13	
Stdev	1.22	2.74	4.99	8.32	1.39	2.45	0.82	
2SE	0.62	1.43	2.83	4.71	0.91	1.81	0.60	
Mortality	5	1	2	0	3	2	1	14
Cumul.Mort.	25	30	40	40	55	65	70	70

Population 05. 20°C								
Total no. egg/week	2	37	62	75	63	24	33	296
Mean egg/ind	0.11	1.95	3.44	4.17	3.50	1.33	2.20	
Cumul. Mean	0.11	2.05	5.50	9.66	13.16	14.50	16.70	
Stdev	0.46	2.53	2.04	3.00	2.83	1.19	2.27	
2SE	0.21	1.14	0.94	1.38	1.31	0.55	1.15	
Mortality	1	0	0	0	0	0	4	5
Cumul.Mort.	5	5	5	5	5	5	25	25
30°C								
Total no. egg/week	58	73	124	56	39	27	24	401
Mean egg/ind	3.4	6.1	13.8	11.2	7.8	6.8	6.0	
Cumul. Mean	3.4	9.5	23.3	34.5	42.3	49.0	55.0	
Stdev	3.7	3.3	3.5	4.9	5.8	5.0	4.5	
2SE	1.8	1.9	2.3	4.3	5.1	4.9	4.5	
Mortality	3	5	3	4	0	1	0	16
Cumul.Mort.	15	40	55	75	75	80	80	80

Population 06. 20°C								
Total no. egg/week	20	67	78	53	45	37	16	316
Mean egg/ind	1.11	3.72	4.59	3.53	3.75	3.70	2.00	
Cumul. Mean	1.11	4.83	9.42	12.95	16.70	20.40	22.40	
Stdev	1.13	1.99	2.15	1.60	1.22	1.70	1.93	
2SE	0.54	0.95	1.02	0.76	0.58	0.81	0.92	
Mortality	2	0	1	2	3	2	2	
Cumul.Mort.	10	10	15	25	40	50	60	60
30°C								
Total no. egg/week	58	54	27	15	7	6	5	172
Mean egg/ind	3.41	3.86	2.70	1.88	1.40	1.50	1.25	
Cumul. Mean	3.41	7.27	9.97	11.85	13.25	14.75	16.00	
Stdev	2.53	1.10	0.67	1.25	1.14	1.29	0.96	
2SE	1.20	0.52	0.32	0.59	0.54	0.61	0.46	
Mortality	3	3	3	2	3	1	1.	
Cumul.Mort.	15	30	45	55	70	75	80	80

Population 07. 20°C								
Total no. egg/week	1	20	17	16	15	4	1	74
Mean egg/ind	0.06	1.43	1.42	1.60	1.88	0.67	0.20	
Cumul. Mean	0.06	1.48	2.90	4.50	6.38	7.04	7.24	
Stdev	0.24	1.50	1.51	1.17	0.99	0.82	0.45	
2SE	0.11	0.79	0.85	0.73	0.69	0.65	0.39	
Mortality	2	4	2	2	2	2	1	15
Cumul.Mort.	10	30	40	50	60	70	75	75
30°C								
Total no. egg/week	21	55	105	87	55	54	38	415
Mean egg/ind	1.31	5.00	10.50	9.67	6.88	7.71	5.43	
Cumul. Mean	1.31	6.31	16.81	26.48	33.35	41.07	46.50	
Stdev	1.89	4.15	4.86	2.96	3.27	4.03	4.61	
2SE	0.92	2.45	3.01	1.93	2.27	2.99	3.42	
Mortality	4	5	1	1	1	1	0	13
Cumul.Mort.	20	45	50	55	60	65	65	65

Population 08. 20°C								
Total no. egg/week	0	1	28	25	22	13	14	103
Mean egg/ind	0.00	0.06	2.33	3.13	3.14	1.86	2.33	
Cumul. Mean	0.00	0.06	2.40	5.52	8.66	10.52	12.85	
Stdev	0.00	0.25	3.14	3.23	2.54	1.46	2.94	
2SE	0.00	0.12	1.78	2.24	1.89	1.08	2.36	
Mortality	3	1	4	4	1	0	1	14
Cumul.Mort.	15	20	40	60	65	65	70	70
30°C								
Total no. egg/week	24	97	96	62	60	39	31	409
Mean egg/ind	1.50	6.47	8.73	6.20	8.57	6.50	5.17	
Cumul. Mean	1.50	7.97	16.69	22.89	31.47	37.97	43.13	
Stdev	2.25	4.07	5.08	3.49	4.96	2.74	2.64	
2SE	1.10	2.06	3.00	2.16	3.68	2.19	2.11	
Mortality	4	1	4	1	3	1	0	14
Cumul.Mort.	20	25	45	50	65	70	70	70

Population 09. 20°C								
Total no. egg/week	1	20	114	76	60	24	24	319
Mean egg/ind	0.05	1.11	6.71	4.47	3.75	1.60	1.71	
Cumul. Mean	0.05	1.16	7.87	12.34	16.09	17.69	19.40	
Stdev	0.22	2.59	2.20	2.21	2.11	1.64	1.27	
2SE	0.10	1.20	1.05	1.05	1.04	0.83	0.66	
Mortality	0	2	1	0	1	1	1	6
Cumul.Mort.	0	10	15	15	20	25	30	30
30°C								
Total no. egg/week	69	140	105	89	81	68	40	592
Mean egg/ind	3.83	9.33	9.55	8.90	8.10	7.56	5.00	
Cumul. Mean	3.83	13.17	22.71	31.61	39.71	47.27	52.27	
Stdev	3.85	6.11	6.09	4.65	3.93	2.13	2.07	
2se	1.78	3.09	3.60	2.88	2.43	1.39	1.43	
Mortality	2	3	4	1	0	1	1	12
Cumul.Mort.	10	25	45	50	50	55	55	55

Population 10 20°C								
Total no. egg/week	0	14	93	108	73	37	20	345
Mean egg/ind	0.00	0.70	4.89	6.00	4.06	2.18	1.25	
Cumul. Mean	0.00	0.70	5.59	11.59	15.65	17.83	19.08	
Stdev	0.00	1.08	2.90	2.25	1.83	1.55	1.00	
2SE	0.00	0.47	1.31	1.04	0.85	0.74	0.49	
Mortality	0	0	1	1	0	1	1	4
Cumul.Mort.	0	0	5	10	10	15	20	20
30°C								
Total no. egg/week	5	54	46	32	32	14	12	195
Mean egg/ind	0.33	4.50	5.11	4.57	4.57	2.33	2.40	
Cumul. Mean	0.33	4.83	9.94	14.52	19.09	21.42	23.82	
Stdev	1.05	3.00	3.52	2.57	3.31	2.07	1.52	
2SE	0.53	1.70	2.30	1.91	2.45	1.65	1.33	
Mortality	5	3	3	2	0	1	1	15
Cumul.Mort.	25	40	55	65	65	70	75	75

Population 11. 20°C								
Total no. egg/week	1	60	66	42	77	50	17	327
Mean egg/ind	0.88	4.29	5.08	4.20	7.70	5.56	2.43	
Cumul. Mean	0.88	5.17	10.25	14.45	22.15	27.71	31.11	
Stdev	1.36	3.00	3.55	3.16	3.95	2.92	1.72	
2SE	0.69	1.52	1.79	1.60	2.00	1.48	0.87	
Mortality	3	3	1	3	0	1	2	
Cumul.Mort.	15	30	35	50	50	55	65	65
30°C								
Total no. egg/week	262	200	152	95	72	57	29	867
Mean egg/ind	14.56	11.76	11.69	9.50	9.00	8.14	4.83	
Cumul. Mean	14.56	26.32	38.01	47.51	56.51	64.65	69.48	
Stdev	6.95	5.38	6.42	3.47	3.25	5.24	2.23	
2SE	3.52	2.72	3.25	1.76	1.65	2.65	1.13	
Mortality	2	1	4	3	2	1	1	
Cumul.Mort.	10	15	35	50	60	65	70	70

Population 12. 20°C								
Total no. egg/week	11	59	68	50	74	60	23	345
Mean egg/ind	0.65	4.21	5.23	4.17	6.73	6.00	3.83	
Cumul. Mean	0.65	4.86	10.09	14.26	20.99	26.99	30.82	
Stdev	1.11	2.99	3.49	3.19	4.17	3.65	1.17	
2SE	0.56	1.51	1.77	1.61	2.11	1.85	0.59	
Mortality	3	3	1	1	1	1	4	
Cumul.Mort.	15	30	35	40	45	50	70	70
30°C								
Total no. egg/week	224	261	225	158	90	59	23	1040
Mean egg/ind	12.44	15.35	13.24	10.53	6.92	4.92	2.88	
Cumul. Mean	12.44	27.79	41.03	51.56	58.48	63.40	66.28	
Stdev	6.35	7.47	5.56	3.98	4.03	3.96	2.03	
2SE	3.21	3.78	2.82	2.01	2.04	2.01	1.03	
Mortality	2.00	1.00	0.00	2.00	2.00	1.00	4.00	
Cumul.Mort.	10.00	15.00	15.00	25.00	35.00	40.00	60.00	60.00

Population 13. 20°C								
Total no. egg/week	3	70	87	82	68	61	55	426
Mean egg/ind	0.15	3.50	4.35	4.32	4.25	4.07	4.23	
Cumul. Mean	0.15	3.65	8.00	12.32	16.57	20.64	24.87	
Stdev	0.49	2.82	4.27	3.86	2.57	2.09	2.35	
2SE	0.25	1.43	2.16	1.95	1.30	1.06	1.19	
Mortality	0	0	0	1	3	1	2	
Cumul.Mort.	0	0	0	5	20	25	35	35
30°C								
Total no. egg/week	227	176	164	134	88	48	23	860
Mean egg/ind	11.95	11.00	13.67	13.40	9.78	6.86	4.60	
Cumul. Mean	11.95	22.95	36.62	50.02	59.80	66.66	71.26	
Stdev	7.70	5.79	7.97	4.22	7.07	5.98	3.78	
2SE	3.90	2.93	4.03	2.14	3.58	3.03	1.91	
Mortality	1	3	4	2	1	2	2	
Cumul.Mort.	5	20	40	50	55	65	75	75

Population 14. 20°C								
Total no. egg/week	14	62	53	54	55	55	27	320
Mean egg/ind	0.78	3.44	3.31	3.86	3.93	4.58	3.00	
Cumul. Mean	0.78	4.22	7.53	11.39	15.32	19.90	22.90	
Stdev	1.11	2.96	2.44	2.60	2.02	2.43	2.18	
2SE	0.56	1.50	1.24	1.31	1.02	1.23	1.10	
Mortality	1	0	2	2	0	2	3	
Cumul.Mort.	5	5	10	30	30	40	55	55
30°C								
Total no. egg/week	134	142	129	92	66	41	20	624
Mean egg/ind	7.44	9.47	9.92	8.36	7.33	5.13	4.00	
Cumul. Mean	7.44	16.91	26.83	35.19	42.52	47.65	51.65	
Stdev	4.13	5.60	6.36	4.52	5.43	3.83	2.00	
2SE	2.09	2.84	3.22	2.29	2.75	1.94	1.01	
Mortality	2.00	3.00	2.00	2.00	2.00	1.00	3.00	
Cumul.Mort.	10.00	15.00	25.00	45.00	55.00	60.00	75.00	75.00

Population 15. 20°C								
Total no. egg/week	19	61	64	53	60	24	9	290
Mean egg/ind	1.06	4.06	4.25	4.00	4.50	2.25	1.29	
Cumul. Mean	1.06	5.12	9.37	13.37	17.87	20.12	21.41	
Stdev	1.76	2.19	1.91	2.20	2.38	1.36	0.76	
2SE	0.89	1.11	0.97	1.12	1.20	0.69	0.38	
Mortality	2	1	1	1	1	1	4	
Cumul.Mort.	10	15	20	25	30	35	55	55
30°C								
Total no. egg/week	49	129	98	62	46	21	11	416
Mean egg/ind	2.88	9.21	8.17	5.64	5.75	3.50	2.75	
Cumul. Mean	2.88	12.09	20.26	25.90	31.65	35.15	37.90	
Stdev	2.93	3.56	3.54	2.91	2.31	1.38	1.26	
2SE	1.48	1.80	1.79	1.47	1.17	0.70	0.64	
Mortality	3	2	2	1	3	2	3	
Cumul.Mort.	15	25	35	40	55	65	80	80

Population 16. 20°C								
Total no. egg/week	4	80	76	57	53	35	22	327
Mean egg/ind	0.24	5.06	6.00	4.38	5.09	3.50	2.75	
Cumul. Mean	0.24	5.30	11.30	15.68	20.77	24.27	27.02	
Stdev	0.75	3.21	3.11	2.10	2.47	2.17	1.83	
2SE	0.38	1.63	1.57	1.06	1.25	1.10	0.93	
Mortality	3	1	3	0	1	1	2	
Cumul.Mort.	15	20	35	35	40	45	65	65
30°C								
Total no. egg/week	99	147	122	85	48	28	15	544
Mean egg/ind	5.53	10.13	9.43	7.38	5.30	4.00	3.00	
Cumul. Mean	5.53	15.66	25.09	32.47	37.77	41.77	44.77	
Stdev	2.65	4.65	3.94	3.33	2.26	2.83	1.00	
2SE	1.34	2.35	1.99	1.69	1.15	1.43	0.51	
Mortality	1	3	2	1	3	3	2	
Cumul.Mort.	5	20	30	35	50	65	75	75

Population 17. 20°C								
Total no. egg/week	0	8	27	16	14	10	7	82
Mean egg/ind	0.00	0.53	1.80	1.00	0.93	0.71	0.58	
Cumul. Mean	0.00	0.53	2.33	3.33	4.26	4.97	5.55	
Stdev	0.00	1.13	2.18	1.26	1.22	1.14	1.00	
2SE	0.00	0.57	1.10	0.64	0.62	0.58	0.50	
Mortality	5	0	0	0	0	1	2	
Cumul.Mort.	25	25	25	25	25	30	40	40
30°C								
Total no. egg/week	34	96	92	70	91	69	35	487
Mean egg/ind	2.00	8.08	12.25	8.75	13.86	11.00	5.71	
Cumul. Mean	2.00	10.08	22.33	31.08	44.94	55.94	61.65	
Stdev	3.02	6.29	3.96	4.03	3.98	3.65	3.15	
2SE	1.53	3.18	2.00	2.04	2.01	1.85	1.59	
Mortality	3	5	4	0	1	0	0	
Cumul.Mort.	15	40	60	60	65	65	65	65

Population 18. 20°C								
Total no. egg/week	6	23	25	20	20	14	10	118
Mean egg/ind	0.30	1.20	1.37	1.17	1.18	0.88	0.71	
Cumul. Mean	0.30	1.50	2.87	4.04	5.22	6.10	6.81	
Stdev	0.66	1.06	0.83	0.99	1.19	0.96	0.73	
2SE	0.33	0.53	0.42	0.50	0.60	0.48	0.37	
Mortality	0	0	1	1	1	1	2	
Cumul.Mort.	0	0	5	10	15	20	30	30
30°C								
Total no. egg/week	0	33	61	24	38	29	13	198
Mean egg/ind	0.00	2.75	6.50	3.11	4.89	3.56	1.75	
Cumul. Mean	0.00	2.75	9.25	12.36	17.25	20.81	22.56	
Stdev	0.00	4.92	3.50	1.90	3.10	2.60	1.28	
2SE	0.00	2.49	1.77	0.96	1.57	1.32	0.65	
Mortality	4	4	2	1	0	0	1	
Cumul.Mort.	20	40	50	55	55	55	60	60

Population 19. 20°C								
Total no. egg/week	0	21	33	32	5	16	7	114
Mean egg/ind	0.00	1.22	1.83	2.12	0.36	1.14	0.54	
Cumul. Mean	0.00	1.22	3.05	5.17	5.53	6.67	7.21	
Stdev	0.00	3.54	1.98	2.76	0.63	1.17	0.78	
2SE	0.00	1.79	1.00	1.40	0.32	0.59	0.39	
Mortality	2	0	0	1	3	0	1	
Cumul.Mort.	10	10	10	15	30	30	35	35
30°C								
Total no. egg/week	1	21	19	13	14	10	7	85
Mean egg/ind	0.07	1.91	2.38	2.60	3.50	2.50	1.75	
Cumul. Mean	0.07	1.98	4.36	6.96	10.46	12.96	14.71	
Stdev	0.26	1.64	1.41	0.55	1.91	1.29	1.26	
2SE	0.13	0.83	0.71	0.28	0.97	0.65	0.64	
Mortality	4	4	3	3	1	0	1	
Cumul.Mort.	20	40	55	70	75	75	80	80

Population 20. 20°C								
Total no. egg/week	14	22	26	18	20	10	15	125
Mean egg/ind	0.74	1.16	1.42	1.06	1.31	0.73	1.15	
Cumul. Mean	0.74	1.90	3.32	4.38	5.69	6.42	7.57	
Stdev	1.28	1.92	0.90	0.73	0.87	0.80	0.80	
2SE	0.65	0.97	0.46	0.37	0.44	0.40	0.41	
Mortality	0	0	0	1	2	1	2	
Cumul.Mort.	0	0	0	5	15	20	30	30
30°C								
Total no. egg/week	13	52	70	40	29	16	19	239
Mean egg/ind	0.72	3.93	5.71	3.46	2.69	1.67	1.91	
Cumul. Mean	0.72	4.65	10.36	13.82	16.51	18.18	20.09	
Stdev	1.07	2.25	3.60	1.33	1.84	1.61	2.77	
2SE	0.54	1.14	1.82	0.67	0.93	0.82	1.40	
Mortality	1	3	1	1	0	1	1	
Cumul.Mort.	5	20	25	30	30	35	40	40

Population 21. 20°C								
Total no. egg/week	11	54	60	35	24	12	6	202
Mean egg/ind	0.61	3.29	4.13	3.00	2.08	1.18	0.88	
Cumul. Mean	0.61	3.89	8.02	11.02	13.10	14.28	15.16	
Stdev	0.92	2.42	2.77	1.47	1.56	1.25	0.99	
2SE	0.46	1.22	1.40	0.74	0.79	0.63	0.50	
Mortality	1	1	2	2	2	1	3	
Cumul.Mort.	5	10	20	30	40	45	60	60
30°C								
Total no. egg/week	75	136	104	55	32	23	7	432
Mean egg/ind	4.76	8.88	6.81	4.00	2.75	2.56	1.40	
Cumul. Mean	4.76	13.64	20.45	24.45	27.20	29.76	31.16	
Stdev	2.91	2.78	2.43	2.18	1.66	2.01	1.67	
2SE	1.47	1.41	1.23	1.11	0.84	1.02	0.85	
Mortality	2	1	0	2	2	3	4	
Cumul.Mort.	10	15	15	25	35	50	70	70

Population 22. 20°C								
Total no. egg/week	0	9	11	22	28	13	14	97
Mean egg/ind	0.00	0.71	0.69	1.38	1.87	0.93	1.08	
Cumul. Mean	0.00	0.71	1.40	2.78	4.65	5.58	6.66	
Stdev	0.00	1.16	0.95	1.54	1.46	1.14	0.76	
2SE	0.00	0.59	0.48	0.78	0.74	0.58	0.38	
Mortality	2	0	1	0	1	1	2	
Cumul.Mort.	10	10	15	15	20	25	35	35
30°C								
Total no. egg/week	107	129	108	86	58	33	27	548
Mean egg/ind	6.71	9.27	7.73	7.08	5.82	4.44	4.71	
Cumul. Mean	6.71	15.98	23.71	30.79	36.61	41.05	45.76	
Stdev	3.55	5.51	3.73	3.45	3.34	2.96	0.95	
2SE	1.80	2.79	1.89	1.75	1.69	1.50	0.48	
Mortality	1	3	0	2	2	3	2	
Cumul.Mort.	5	20	20	40	50	65	75	75

Population 23. 20°C								
Total no. egg/week	3	12	15	12	5	5	3	55
Mean egg/ind	0.19	0.80	1.29	1.00	0.38	0.42	0.30	
Cumul. Mean	0.19	0.99	2.28	3.28	3.66	4.08	4.38	
Stdev	0.54	1.32	1.20	1.08	0.51	0.79	0.67	
2SE	0.28	0.67	0.61	0.55	0.26	0.40	0.34	
Mortality	2	1	1	1	1	1	2	
Cumul.Mort.	10	15	20	25	30	35	45	45
30°C								
Total no. egg/week	5	20	36	78	53	22	11	225
Mean egg/ind	0.28	1.63	2.71	6.54	4.38	2.00	1.70	
Cumul. Mean	0.28	1.91	4.62	11.16	15.54	17.54	19.24	
Stdev	0.75	2.03	2.73	3.71	2.87	1.71	1.95	
2SE	0.38	1.03	1.38	1.88	1.45	0.86	0.99	
Mortality	1	2	2	1	0	1	2	
Cumul.Mort.	5	15	25	30	30	35	45	45

Population 24. 20°C								
Total no. egg/week	5	75	35	24	22	27	8	196
Mean egg/ind	0.29	6.14	2.93	2.08	1.85	2.31	0.75	
Cumul. Mean	0.29	6.43	9.36	11.44	13.29	15.60	16.35	
Stdev	0.69	2.54	1.98	1.38	1.82	2.50	0.87	
2SE	0.35	1.28	1.00	0.70	0.92	1.26	0.44	
Mortality	1	3	0	1	0	0	1	
Cumul.Mort.	5	20	20	25	25	25	30	30
30°C								
Total no. egg/week	37	137	92	55	44	25	13	403
Mean egg/ind	2.69	9.13	7.08	4.23	3.38	1.92	1.30	
Cumul. Mean	2.69	11.82	18.90	23.13	26.51	28.43	29.73	
Stdev	2.87	3.93	3.71	2.13	1.89	1.55	1.49	
2SE	1.45	1.99	1.88	1.08	0.96	0.79	0.76	
Mortality	2	1	2	0	0	0	3	
Cumul.Mort.	10	25	35	35	35	35	50	50

Population 25. 20°C								
Total no. egg/week	6	31	35	15	21	5	7	120
Mean egg/ind	0.35	2.20	2.86	1.14	1.50	0.36	0.50	
Cumul. Mean	0.35	2.55	5.41	6.55	8.05	8.41	8.91	
Stdev	0.61	2.48	2.51	1.41	1.09	0.63	0.76	
2SE	0.31	1.26	1.27	0.71	0.55	0.32	0.38	
Mortality	1	2	1	0	0	0	0	
Cumul.Mort.	5	15	20	20	20	20	20	20
30°C								
Total no. egg/week	13	76	86	53	31	18	6	283.00
Mean egg/ind	0.93	5.07	5.87	4.08	2.38	1.80	0.60	
Cumul. Mean	0.93	6.00	11.87	15.95	18.33	20.13	20.73	
Stdev	1.58	3.83	3.50	4.70	2.90	1.81	0.97	
2SE	0.80	1.94	1.77	2.38	1.47	0.92	0.49	
Mortality	2	0	0	2	0	3	0	
Cumul.Mort.	10	10	10	20	20	35	35	35

Population 26. 20°C								
Total no. egg/week	20	28	23	23	16	7	6	123
Mean egg/ind	1.25	2.15	1.77	2.09	1.78	1.00	0.86	
Cumul. Mean	1.25	3.40	5.17	7.26	9.04	10.04	10.90	
Stdev	1.18	2.30	1.54	1.97	1.09	0.82	0.69	
2SE	0.60	1.17	0.78	1.00	0.55	0.41	0.35	
Mortality	2	3	0	2	2	2	2	
Cumul.Mort.	10	25	25	35	45	55	65	65
30°C								
Total no. egg/week	160	164	106	84	63	51	28	65
Mean egg/ind	10.38	11.13	7.60	6.00	5.00	3.93	2.29	
Cumul. Mean	10.38	21.51	29.11	35.11	40.11	44.04	46.33	
Stdev	3.40	4.17	3.36	4.07	3.44	3.24	2.46	
2SE	1.72	2.11	1.70	2.06	1.74	1.64	1.25	
Mortality	2	1	1	0	0	0	1	
Cumul.Mort.	10	15	20	20	20	20	25	25

Population 27. 20°C								
Total no. egg/week	11	16	22	18	16	12	4	99
Mean egg/ind	0.61	1.00	1.47	1.43	1.14	1.00	0.40	
Cumul. Mean	0.61	1.61	3.08	4.51	5.66	6.65	7.05	
Stdev	0.78	1.17	1.25	0.94	1.10	0.68	0.52	
2SE	0.39	0.59	0.63	0.47	0.56	0.29	0.26	
Mortality	1	1	2	1	0		3	
Cumul.Mort.	5	10	20	25	25	30	45	45
30°C								
Total no. egg/week	27	70	76	58	52	37	15	335
Mean egg/ind	1.69	4.87	5.71	4.77	4.23	3.55	1.78	
Cumul. Mean	1.69	6.56	12.27	17.07	21.27	24.82	26.60	
Stdev	1.49	1.96	3.52	2.92	2.39	1.44	1.09	
2SE	0.76	0.99	1.78	1.48	1.21	0.73	0.55	
Mortality	2	1	1	1	0	2	2	
Cumul.Mort.	10	15	20	25	25	30	40	40

Population 28. 20°C								
Total no. egg/week	2	3	12	12	11	9	5	54
Mean egg/ind	0.12	0.21	1.00	1.17	1.20	1.00	0.63	
Cumul. Mean	0.12	0.33	1.33	2.50	3.70	4.70	5.33	
Stdev	0.33	0.58	0.85	0.58	1.03	0.67	0.52	
2SE	0.17	0.29	0.43	0.29	0.52	0.34	0.26	
Mortality	1	3	3	1	2	0	2	
Cumul.Mort.	5	20	35	40	50	50	60	60
30°C								
Total no. egg/week	73	125	115	71	56	26	16	482
Mean egg/ind	4.22	8.00	7.93	5.69	4.92	2.80	1.80	
Cumul. Mean	4.22	12.22	20.15	25.84	30.76	33.56	35.36	
Stdev	3.81	2.83	3.15	2.02	2.71	1.23	1.23	
2SE	1.93	1.43	1.59	1.02	1.37	0.62	0.62	
Mortality	1	2	1	2	1	3	0	
Cumul.Mort.	5	15	20	30	35	45	45	45

Population 29. 20°C								
Total no. egg/week	1	6	12	14	23	14	14	84
Mean egg/ind	0.06	0.38	0.81	0.94	1.56	1.14	1.15	
Cumul. Mean	0.06	0.44	1.25	2.19	3.75	4.89	6.04	
Stdev	0.24	0.89	0.91	0.77	0.73	0.86	0.80	
2SE	0.12	0.45	0.46	0.39	0.37	0.44	0.41	
Mortality	1	1	0	0	0	2	2	
Cumul.Mort.	5	10	10	10	10	20	40	40
30°C								
Total no. egg/week	45	77	63	41	28	14	7	275
Mean egg/ind	3.75	5.44	4.71	3.31	2.42	1.70	1.13	
Cumul. Mean	3.75	9.19	13.90	17.21	19.63	21.33	22.46	
Stdev	4.77	2.22	1.82	1.84	1.51	1.16	0.83	
2SE	2.41	1.12	0.92	0.93	0.76	0.59	0.42	
Mortality	3	0	2	1	1	2	3	
Cumul.Mort.	15	15	25	30	35	45	60	60

Population 30. 20°C								
Total no. egg/week	0	6	20	12	14	11	5	68
Mean egg/ind	0.00	0.38	1.40	0.93	1.25	1.20	0.63	
Cumul. Mean	0.00	0.38	1.78	2.71	3.96	5.16	5.79	
Stdev	0.00	0.72	0.99	0.80	0.87	0.63	0.52	
2SE	0.00	0.36	0.50	0.40	0.44	0.32	0.26	
Mortality	3	1	1	0	3	2	2	
Cumul.Mort.	15	20	25	25	40	50	60	60
30°C								
Total no. egg/week	68	101	65	47	23	21	15	340
Mean egg/ind	4.35	6.56	4.93	4.08	2.36	2.56	2.43	
Cumul. Mean	4.35	10.91	15.84	19.92	22.28	24.84	27.27	
Stdev	4.14	3.31	1.69	1.80	1.43	2.13	1.27	
2SE	2.09	1.67	0.85	0.91	0.73	1.08	0.64	
Mortality	2	1	2	1	2	2	3	
Cumul.Mort.	10	15	25	30	40	50	65	65